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(57) Abstract

The present invention provides compositions and methods for the collection, cryopreservation, and transfusion of human peripheral blood cells, and in particular, granulocytes. Within one aspect of the invention compositions are provided comprising frozen granulocytes in a cryopreservation medium, the granulocytes having a biological activity upon thawing of at least 30 % as compared to fresh donor granulocytes in an intracellular killing assay, or alternatively, at least about 10 % of their respiratory burst activity as compared with fresh granulocytes in a respiratory burst activity assay. Within other aspects, methods are provided for preventing or treating fever or infection in a patient during a period of risk for fever or infection, comprising the steps of: (a) harvesting granulocytes from the blood of a patient or donor by apheresis; (b) freezing the harvested granulocytes in a cryopreservation medium, such that, upon thawing, the granulocytes have a biological activity of at least 30 % as compared to fresh granulocytes in an intracellular killing assay, or alternatively, at least about 10 % of their respiratory burst activity as compared with fresh granulocytes in a respiratory burst activity assay; (c) thawing the frozen granulocytes and (d) administering a therapeutically effective amount of thawed granulocytes to a patient during a period of risk for fever or infection, such that the fever or infection may be prevented or reduced.

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Description

METHODS FOR COLLECTION AND CRYOPRESERVATION OF HUMAN GRANULOCYTES

Technical Field

This invention relates to transfusion therapy and, more specifically, to methods for the collection, cryopreservation, and transfusion of human peripheral blood cells, especially granulocytes.

Background of the Invention

Granulocytes are bone marrow-derived white blood cells of the myeloid lineage. They are commonly divided into neutrophils, eosinophils, and basophils, according to the type of dye with which the granules in their cytoplasm stain. The majority of circulating granulocytes are neutrophils (also called polymorphonuclear cells or PMNs). Human blood typically contains between 1500 and 7000 neutrophils/μL (1.5-7.0 x 10^9/L).

Granulocytes function as phagocytic cells, killing or inactivating pathogenic microorganisms, especially pyogenic (pus-forming) bacteria. When the number of granulocytes is low (granulocytopenia) or when they are functionally abnormal (granulocyte dysfunction), the incidence and severity of bacterial infections in the host is markedly increased (Bodey et al., *Ann. Intern. Med. 64*:328-340, 1966). A patient is generally considered to be granulocytopenic when his blood granulocyte count falls below about 1000 cells/μL, for example, secondary to cancer chemotherapy or bone marrow failure. In addition to quantitative defects in granulocyte function, various qualitative defects have been described as well, such as chronic granulomatous disease, in which cell numbers are approximately normal, but functional activity is depressed.

Granulocytopenia may be treated with cytokines, such as granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage-colony stimulating factor (GM-CSF), or interleukin-1 (IL-1), or by transfusion (Strumia, *Am. J. Med. Sci. 187*:527-544, 1934; Dreyfus, *Sang 19*:570-574, 1948; Hirsch and Gardner, *J. Lab. Clin. Med. 39*:556-569, 1952; Morse et al., *Transfusion 6*:183-192, 1966). Cytokines act slowly (over a period of days to weeks) to expand the number of circulating granulocytes; hence, the patient may remain at risk of infection for several days after therapy is commenced (reviewed in

Moore, *Cancer 67*:2718-2726, 1991). Furthermore, cytokines are expensive and may cause undesirable side effects.

Transfusion therapy, on the other hand, results in an immediate increase in the level of circulating granulocytes and hence an immediate diminution in risk of infection, provided a sufficient dose of biologically active granulocytes is given. However, it is notoriously difficult to harvest granulocytes in sufficient numbers for transfusion. Ideally, a granulocyte transfusion should deliver 10^{10} granulocytes to an adult patient or 10^9 granulocytes/kg body weight to a neonate to achieve an increment of 1000 granulocytes/μL in the peripheral circulation (Herzig, in Garratty (ed.), *Current Concepts in Transfusion Therapy*, Arlington, VA: American Assoc. of Blood Banks, pp. 267-294, 1985). Granulocytes have a relatively short half-life in the circulation (about 5-7 hours), hence daily transfusions may be required to maintain the increment achieved by the initial transfusion.

Granulocyte collection and transfusion is further confounded by the difficulty of preserving granulocytes in a biologically active state for more than a matter of hours. Room temperature storage has been reported to be better than refrigerated storage, but even at room temperature, there is marked loss of functional activity within 8-24 hours (*see* Herzig, 1985, ibid.; McCullough, in Garratty (ed.), *Current Concepts in Transfusion Therapy*, Arlington, VA: American Assoc. Blood Banks, pp. 125-181, 1985).

Cryopreservation (freezing) of granulocytes has been even less successful (reviewed in Bank, *Cryobiology 17*:187-197, 1980; Takahashi et al., *Cryobiology 22*:215-236, 1985; Yang et al., *Crybiology 29*:500-510, 1992). Consequently, allogeneic (donor) granulocyte transfusions have been the only possible option, despite the drawbacks associated with allogeneic transfusions, such as the potential for transmission of disease, alloimmunization, and graft-versus-host disease. Even donor granulocyte transfusions are uncommon owing to the difficulty of obtaining sufficient quantities of cells to support a granulocytopenic patient.

Accordingly, there exists a need in the art of transfusion practice for a method of collecting and cryopreserving granulocytes for transfusion therapy of granulocytopenia, which method preserves cell viability and biological activity. In particular, there is a need for a clinically practicable method of collecting and preserving autologous (self) granulocytes. The present invention provides such methods, and further, provides other related advantages.

Summary of the Invention

Briefly stated, the present invention provides compositions and methods for the collection, cryopreservation, and transfusion of human peripheral blood cells, and in particular, granulocytes.

More specifically, within one aspect of the present invention, compositions are provided comprising frozen granulocytes in a cryopreservation medium, the granulocytes having a biological activity upon thawing of at least about 30% as compared to fresh donor granulocytes in an intracellular killing assay, or alternatively, at least about 10% of their respiratory burst activity as compared with fresh granuloyctes in a respiratory burst activity assay. Within another aspect, compositions are provided comprising frozen mobilized granulocytes in a cryopreservation medium, the granulocytes having a biological activity upon thawing of at least 30% as compared to fresh donor granulocytes in an intracellular killing assay, or alternatively, at least about 10% of their respiratory burst activity as compared with fresh granuloyctes in a respiratory burst activity assay. Within preferred embodiments, the above granulocytes have a biological activity upon thawing of at least about 50% as compared to fresh donor granulocytes in an intracellular killing assay.

Within other aspects of the invention, methods of preserving granulocytes are provided comprising the steps of (a) harvesting granulocytes from the blood of a patient or donor by apheresis, and (b) freezing the harvested granulocytes in a cryopreservation medium, such that, upon thawing, the granulocytes have a biological activity of at least about 30% as compared to fresh granulocytes in an intracellular killing assay, or alternatively, at least about 10% of their respiratory burst activity as compared with fresh granuloyctes in a respiratory burst activity assay. Within one embodiment, the granulocytes are harvested in the presence of a red cell sedimenting agent selected from the group consisting of hydroxyethyl starch, pentastarch, fluid gelatin and dextran.

Within another aspect of the invention, methods are provided for preventing or treating fever or infection in a patient during a period of risk for fever or infection, comprising the steps of (a) harvesting granulocytes from the blood of a patient or donor by apheresis, (b) freezing the harvested granulocytes in a cryopreservation medium, such that, upon thawing, the granulocytes have a biological activity of at least about 30% as compared to fresh granulocytes in an intracellular killing assay, or alternatively, at least about 10% of their respiratory burst activity as compared with fresh granuloyctes in a respiratory burst activity assay, (c) thawing the frozen granulocytes, and (d) administering a

therapeutically or prophylactically effective amount of thawed granulocytes to a patient during a period of risk for fever or infection, such that the fever or infection may be prevented or reduced. Within certain embodiments of the present invention, the patient is a patient with granulocyte dysfunction or granulocytopenia. Examples of patients with granulocytopenia or granulocyte dysfunction include patients with an idiopathic or hereditary defect in granulocyte number or function, bone marrow transplant patients, peripheral blood stem cell transplant patients, patients on a myelosuppressive chemotherapeutic or radiotherapeutic regimen, patients on an immunosuppressive regimen, patients with sepsis, patients with immune deficiency disease, and patients who have experienced blood loss.

Within certain embodiments of the invention, the patient may also be administered a composition selected from the group consisting of antibiotics, antifungals, antivirals, and antiprotozoals. Within other embodiments of the invention, prior to the step of harvesting granulocytes, the granulocytes in a patient or donor may be mobilized.

As utilized herein and as described in more detail below, the phrase mobilized granulocytes refers to those granulocytes which have been obtained from a patient exposed to a drug which acts on myeloid cells and/or their progenitors. Within one embodiment, granulocytes may be mobilized in a patient or donor by administration of cytokine to the patient or donor. Preferably, the cytokine is selected from the group consisting of G-CSF, GM-CSF, IL-3, SCF and IL-1. Within additional embodiments, the granulocytes are mobilized in a patient or donor by administration of a steroid. Within yet other embodiments, the granulocytes are harvested in the presence of a red cell sedimenting agent selected from the group consisting of hydroxyethyl starch, pentastarch, fluid gelatin and dextran. Within other embodiments, the granulocytes are either allogeneic, syngeneic or autologous to the patient.

Within a particularly preferred embodiment of the invention, methods are provided for treating or preventing fever or infection in a patient during a period of risk for fever or infection, comprising the steps of (a) mobilizing granulocytes in a patient or donor, (b) harvesting granulocytes from the patient or donor by apheresis in the presence of a red cell sedimenting agent, (c) freezing the harvested granulocytes in a cryopreservation medium, such that, upon thawing, the granulocytes have a biological activity of at least about 30% as compared to fresh granulocytes in an intracellular killing assay, or alternatively, at least about 10% of their respiratory burst activity as compared

with fresh granuloyctes in a respiratory burst activity assay, (d) thawing the frozen granulocytes, and (e) administering a therapeutic or prophylactically effective amount of thawed granulocytes to a patient during a period of risk for fever or infection, such that the fever or infection may be treated or prevented.

Within other aspects of the invention, granulocytes characterized as having been prepared by the following process are provided, the process comprising the steps of (a) harvesting granulocytes from the blood of a patient or donor by apheresis, (b) freezing the harvested granulocytes in a cryopreservation medium, such that, upon thawing, the granulocytes have a biological activity of at least about 30% as compared to fresh granulocytes in an intracellular killing assay, or alternatively, at least about 10% of their respiratory burst activity as compared with fresh granuloyctes in a respiratory burst activity assay, and (c) thawing the frozen granulocytes. Within one embodiment, the granulocytes are harvested in the presence of a red cell sedimenting agent selected from the group consisting of hydroxyethyl starch, pentastarch, fluid gelatin and dextran. Within preferred embodiments of the invention, granulocytes which are harvested in accordance with the above methods may be additionally partially purified.

Within other embodiments of the above-described compositions and methods, the granulocytes are frozen at a concentration of between 250×10^6 and 500×10^6 cells/mL. Within other embodiments, the granulocytes are irradiated prior to freezing.

Within other aspects of the present invention, a cryopreservation medium is provided comprising a penetrating cryoprotectant, an anticoagulant, and/or a nuclease. Within further embodiments, the cryopreservation media also comprises a physiologically acceptable medium and a source of protein. Examples of physiologically acceptable media include phosphate buffered saline, RPMI, DMEM, and medium 199. An example of a penetrating cryoprotectant is DMSO, which is preferably utilized at a concentration of about 5% to about 10% (by volume), and more preferably, at a concentration of about 7.5%. An example of a source of protein is human serum albumin. Examples of anticoagulants suitable for use with the present invention include acid citrate dextrose, EDTA and heparin. When heparin is utilized as the anticoagulant, it is preferably present at a concentration of between about 50 and about 150 units/mL, and most preferably at a concentration of about 100 units/mL. Examples of nucleases include RNase and DNase, with type I or type II DNases being preferred, and recombinant human DNase being most preferred. In

addition, within preferred embodiments the DNase is present at a concentration of between about 50 and about 150 units/mL, and most preferably, at a concentration of about 100 units/mL.

Within another aspect of the invention, compositions are provided comprising (a) white cells (leukocytes) which are at least about 50% granulocytes, the granulocytes having a biological activity of at least about 30% as compared to fresh granulocytes, or alternatively, at least about 10% of their respiratory burst activity as compared with fresh granuloyctes in a respiratory burst activity assay, (b) a penetrating cryoprotectant, and (c) a nuclease. Within various embodiments, the composition may further comprise an anticoagulant, or heparin. Within other embodiments, the penetrating cryoprotectant is DMSO which is preferably present at a concentration of between about 5% and about 10%. Within other embodiments, the compositions further comprise a red cell sedimenting agent, and the nuclease is a DNase.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth below which describe in more detail certain compositions and methods, and are therefore incorporated by reference in their entirety.

Brief Description of the Drawings

Figure 1 provides a dot plot, obtained by fluorescence activated cell sorting (flow cytometry) analysis, of forward angle light scatter (X-axis) versus side scatter (Y-axis) for fresh (unfrozen) granulocytes, isolated by density gradient centrifugation in Ficoll, followed by hemolysis in ammonium chloride.

Figure 2 provides a histogram comparing percent cell loss, post-thaw, when granulocytes are processed prior to freezing by density gradient centrifugation followed by hemolysis, dextran sedimentation, or hetastarch sedimentation, using two different ratios of starch to cells, and frozen in complete cryopreservation medium (panel a) with percent cell loss, post-thaw, when granulocytes processed in the same way prior to freezing are frozen in incomplete cryopreservation medium (panel b).

Figure 3 provides a dot plot, obtained by flow cytometry, of forward angle light scatter (X-axis) versus side scatter (Y axis) (panel a) and of fluorescence intensity (Y-axis) versus forward angle light scatter (X-axis) (panel b) for post-thaw granulocytes collected and cryopreserved in accordance with the methods of this invention.

Detailed Description of the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to first set forth definitions of certain terms that will be used hereinafter.

"Granulocyte" refers to bone marrow-derived white blood cells of the myeloid lineage. Within the context of the present invention, the term "granulocyte" is understood to include neutrophils, polymorphonuclear cells, basophils, and eosinophils. As used herein, the term granulocyte includes not only mature granulocytes but immature forms as well, such as bands, promyelocytes, metamyelocytes, myelocytes, and myeloblasts, that is, all myeloid committed progenitor cells. Granulocytes are considered to be "partially purified" within the context of the present invention if they constitute approximately 50%, more often 70%, by number of the white cells (leukocytes) in a suspension of cells. Other cell types (contaminating cells) present in a partially purified preparation of granulocytes may include other leukocytes (such as lymphocytes, monocytes, and macrophages), erythrocytes, and platelets.

"Mobilization" refers to the migration of hematopoietic precursor cells (stem cells and committed progenitors) from the bone marrow to the peripheral circulation in response to a stimulus, typically a drug such as a steroid, a cytokine, a myelotoxic agent or any combination thereof.

"Biological activity" is used to denote a constellation of functions which are characteristic of a specific cell type. For example, the biological activity exhibited by granulocytes includes locomotion, dye reduction, chemotaxis, respiratory burst activity, plastic adherence, phagocytosis, antibody-mediated cellular cytotoxicity, and intracellular killing, among other functions. As used herein, "intracellular killing" refers to the ability of granulocytes to ingest and destroy bacteria.

"Granulocytopenia" refers to any condition, regardless of etiology, in which there is a reduction in the number and/or function of granulocytes. Granulocytopenia may result from myelotoxic chemotherapy (*e.g.*, cytoxan therapy), radiation therapy, immunosuppressive therapy (*e.g.*, steroid therapy), accidental radiation exposure, immunosuppression due to an infectious agent (*e.g.*, HIV infection), blood loss due to trauma or surgery, idiopathic and hereditary defects in granulocyte number and function (*e.g.*, chronic granulomatous disease), malignancy, and thermal burns, among other possible etiologies.

As noted above, the present invention provides compositions and methods for preventing or treating fever or infection in a patient during a period of risk for fever or infection. Within one aspect of the present invention, methods are provided comprising the steps of (a) harvesting granulocytes from the blood of a patient or donor, (b) freezing the harvested granulocytes in a cryopreservation medium, such that, upon thawing, the granulocytes have a biological activity of at least 30% as compared to fresh donor granulocytes in an intracellular killing assay, (c) thawing the frozen granulocytes, and (d) administering a therapeutically or prophylactically effective amount of thawed granulocytes to a patient during a period of risk for fever or infection, such that the fever or infection may be prevented or reduced.

Granulocyte Harvesting Techniques

Granulocytes may be harvested from the blood by any of several methods, depending upon the volume desired and the equipment available. For example, within one embodiment of the invention, when small volumes of blood are desired, they may be collected directly by phlebotomy.

Alternatively, when larger volumes of blood are required an apheresis procedure may be employed. In apheresis anti-coagulated blood is separated into its components by continuous- or intermittent-flow centrifugation, with certain fractions being returned to the donor or patient while other fractions are saved (reviewed in Herzig, 1985, *op. cit.*). Several apheresis machines are commercially available and may be utilized in the context of the present invention, including the Fenwal CS-3000 (Baxter Healthcare, Chicago, IL), the Cobe 2997 (Cobe BCT, Lakewood, CO), the Cobe Spectra, the Cobe 2991, and the Haemonetics V50 (Haemonetics Corp., Braintree, MA), among other manufacturers and models. Each of these machines can be utilized in accordance with its manufacturer's instructions to prepare various cellular fractions, such as a granulocyte fraction, from peripheral blood.

Generally, granulocytes may be collected by apheresis of from about 1 to 12 units of blood, more often about 4 to 10 units of blood, and typically about 5-7 units of blood. The efficiency of granulocyte collection may be increased by including a red cell sedimenting agent in the apheresis procedure. Red cell sedimenting agents include dextran, fluid gelatin, and hydroxyethyl starch (HES or hetastarch). The latter is a particularly preferred red cell sedimenting agent and is commercially available in two forms, HESPAN and a rapidly excreted, low molecular weight analog, pentastarch

(PENTASPAN), from DuPont-Merck Pharmaceuticals (Wilmington, DE). HES is also available from Fresenius (Germany) under the trade name PLASMASTERIL. Within the context of the instant invention, the term hydroxyethyl starch or HES is used to denote either or both hetastarch and pentastarch.

Within one embodiment of the invention, HES is mixed aseptically with an anticoagulant, usually trisodium citrate, at a ratio of approximately 500 mL of 10% HES to about 30 mL of 46.7% citrate, although both higher and lower amounts of HES can be used satisfactorily (Strauss et al., *J. Clin. Apheresis 6*:241-243, 1991; Strauss et al., *Transfusion 27*:350-352, 1987). The ratio of donor blood to HES may vary from approximately 9:1 to 20:1, more often approximately 13:1 to 18:1.

In another embodiment of the invention, PENTASPAN is mixed aseptically with a commercially available trisodium citrate buffer, such as TRI-CITROL, at a ratio of 500 mL PENTASPAN to 27 mL TRI-CITROL. Within this embodiment, the ratio of blood to PENTASPAN may vary from about 5:1 to 10:1.

The number of units of donor blood processed by apheresis will also vary, depending on premedications, the age and health of the donor or patient, as well as on the number of granulocytes which it is desired to collect. Typically, about 4 to 12 units of donor or patient blood are processed per apheresis, more often 5 to 7 units. Typically, the blood is anticoagulated using ACD-A, although it will be apparent that other anticoagulants may also be used.

A typical apheresis protocol for the isolation of granulocytes using the Cobe Spectra is given below: Donor blood is collected in the presence of HES (500 mL of 6-10% HES (in 0.9% NaCl) to 40 mL of citrate), the ratio of donor blood to HES being about 13:1. The centrifuge is operated at a speed of 603-711 rpm at an inlet flow rate of 44-60 mL/min. Granulocytes are collected at the rate of 3.0 mL/min. Similar protocols have been described in the literature for other apheresis machines (see, for example, Strauss et al., *op. cit.*, 1991, for protocols for the Fenwal CS-3000 and the Cobe 2997, and Caspar et al., *Blood 81*:2866, 1993, for the CS-3000).

Other apheresis products may also be utilized as a source of granulocytes. For example, a mononuclear cell (MNC) fraction may be collected from a mobilized patient (see below) by apheresis and granulocytes isolated therefrom. Within one embodiment, this MNC fraction may be subjected to immunoselection in order to enrich the proportion of cells in the

product which are stem cells, for example, using antibody to the CD34 antigen (Heimfeld et al., *Transplant Proc. 24*:2818, 1992; *see also* U.S. Patent Nos. 5,215,927; 5,225,353 and 5,262,334). The fraction remaining after CD34 selection, *i.e.*, the CD34-fraction, may be used as a source of granulocytes for cryopreservation, according to the methods of the instant invention. Because the proportion of cells in the CD34-fraction of a leukapheresis product which is granulocytes is typically lower than the fraction of cells in a granulocytapheresis product, it may be desirable to further enrich the granulocytes therefrom, for example, by the methods described below.

It may also be possible to collect a combined MNC/granulocyte fraction from peripheral blood by apheresis. Bone marrow is another potential source of granulocytes. The pheresis or marrow product may be derived from a mobilized patient or donor (see below).

When autologous granulocytes are to be used for transfusion, it may be desirable to purge (deplete) the granulocyte product of tumor cells prior to cryopreservation, for example, if the patient is known to have underlying malignant disease which may involve the peripheral blood. Methods of tumor cell purging have been described in the literature and include, for example, pharmacological, immunological, and biophysical methods, as well as combinations thereof. In general, immunological methods are preferred, especially methods which do not employ complement, such as immunoselection methods, in order to minimize bystander damage to the granulocytes. Purging methods are described in the following references: S. Gross, in *Bone Marrow Purging and Processing*, Gross et al. (eds.) New York: Wiley, p. xxix, 1990; E. Ball, in *Bone Marrow Processing and Purging*, AP Gee (ed.), Boca Raton: CRC Press, p. 281, 1991; F. Sieber, in *Bone Marrow Processing and Purging*, AP Gee (ed.), Boca Raton: CRC Press, p. 263, 1991; E.J. Schpall et al., in *Bone Marrow Processing and Purging*, AP Gee (ed.), Boca Raton: CRC Press, p. 307, 1991; and S.D. Rowley and J.M. Davis, in *Bone Marrow Processing and Purging*, AP Gee (ed.), Boca Raton: CRC Press, p. 247, 1991. Alternatively, irradiation, for example, at least about 2500 rads from an electron beam source such as a linear accelerator, may be adequate to purge tumor cells from a granulocyte concentrate.

Granulocyte Mobilization

In order to increase the number of circulating granulocytes in the donor and hence the efficiency of harvest, within various embodiments of the

invention donors may be treated prior to apheresis with a granulocyte mobilizing agent, for example, steroids, such as prednisone (Strauss et al., 1991, *op. cit.*), dexamethasone (Higby et al., *Blood 50*:953, 1977), etiocholanolone or hydrocortisone, among others. For example, approximately 60 mg of prednisone may be administered orally to the donor in divided doses of about 20 mg each, approximately 18, 12, and 4 hours prior to apheresis, or 4 mg/m^2 of dexamethasone intravenously prior to apheresis.

Other methods of granulocyte mobilization may also be utilized within the context of the present invention, including for example the administration of cytokines such as G-CSF (Bensinger et al., *Blood 81*:3158, 1993; Caspar et al., *op. cit.*), GM-CSF (Gianni et al., *Lancet 66*:580, 1989), IL-1, IL-3 and/or SCF. Recombinant human G-CSF and SCF are available from Amgen, Inc. (Thousand Oaks, CA), GM-CSF, IL-1 and PIXYKINE™ from Immunex Corp. (Seattle, WA), and IL-3 from Sandoz. Dosing and schedule of administration may be determined empirically. For example, G-CSF is typically administered subcutaneously at a dose of about 4-20 μg/kg/day or, alternatively, 300 μg/day until pre-donation granulocyte levels exceed 1 x 10^9 cells/L on two successive days, at which time the donor is apheresed. Alternatively, donors may be mobilized by administration of a single 5 ug/kg dose of G-CSF 24 hours prior to apheresis. Other dosing schedules may also be employed and are described in Example 7 of the instant specification.

Cytokine-induced mobilization of granulocytes is advantageous since it also results in the mobilization of immature granulocyte precursors which may have a longer half-life in the circulation than mature cells, thus necessitating fewer transfusions or allowing the interval between transfusions to be lengthened.

Granulocyte mobilization may also be accomplished using a combination of mobilizing agents, for example, a steroid and a cytokine. Myelotoxic agents such as cyclophosphamide (cytoxan, at 3-7 g/m^2; Siena et al., *Blood 74*:1905, 1989), and 5-fluorouracil (5-FU), are also known mobilizing agents and may be used alone or in combination with another mobilizing agent (see, for example, AU-A-23587/88, published 12/04/90).

Within a related embodiment of the invention, the efficiency of granulocyte collection may be enhanced by harvesting granulocytes from donors with chronic myelogenous leukemia (CML), who have elevated levels of circulating granulocytes as a consequence of their underlying disease (Morse et al., *Transfusion 6*:183, 1966; Schiffer et al., *Am. J. Hematol. 15*:65, 1983).

Partial Purification of Granulocytes

A granulocyte fraction obtained by apheresis or phlebotomy may contain other cell types as well as granulocytes. Typically, a granulocyte apheresis product contains white cells which are approximately 50%-85% granulocytes (by cell number), most often approximately 70%-75% granulocytes. Within certain embodiments of the invention, it may be desirable to further purify granulocytes from this fraction by any of a variety of methods, such as hemolysis, density gradient centrifugation, plastic adherence, and/or immunoselection.

Briefly, a wide variety of polymers may be utilized in order to partially purify granulocytes by density gradient centrifugation. These include Ficoll-Isopaque, Ficoll-Hypaque, and Percoll, among others. Ficoll is a high molecular weight sucrose polymer which is mixed with sodium metrizoate (Isopaque) or sodium diatrizoate (Hypaque) to prepare specific density solutions useful for cell separations. Percoll, on the other hand, is a solution of colloidal silica particles coated with polyvinylpyrrolidone (PVP). Density gradients can be continuous, by which is meant that they have the same density throughout, or discontinuous, meaning that the density varies at different points within the gradient. In a discontinuous gradient, the density can vary linearly or non-linearly, *i.e.*, in discrete steps. Density gradients suitable for the purification of granulocytes are discussed in Clay and Kline, in Garratty (ed.), *Current Concepts in Transfusion Therapy*, Arlington, VA: American Assoc. Blood Banks, pp. 183-265, 1985.

In one embodiment of the instant invention, granulocytes obtained by apheresis in the presence of hydroxyethyl starch are partially purified by one or more density gradient centrifugations. For example, in density gradient centrifugation, the granulocyte fraction is layered onto a discontinuous Ficoll-Hypaque (Sigma, St. Louis, MO) gradient, consisting of a lower layer of Ficoll-Hypaque having a specific gravity of 1.119 and an upper layer having a specific gravity of 1.077. The gradient, overlaid with the granulocytapheresis product, is centrifuged for 15 minutes at 1650 x g, and the cell layer at the interface between the two Ficoll layers is harvested. Alternatively, a discontinuous Percoll gradient can be used in which the lower layer has a specific gravity of 1.100; the upper layer, a specific gravity of 1.075; and the gradient, overlaid with granulocytes, is centrifuged at 400 x g for 20 minutes. The granulocytes from the interface of the two Percoll layers are harvested.

Granulocytes can also be partially purified by hemolysis, where the contaminating cells are predominantly red cells. For example, the granulocytes may be suspended in hemolytic buffer (8.3 g/L NH_4Cl, 1.0 g/L $NaHCO_3$, 0.4 g/L EDTA, 10 mLs packed cells to 30 mLs hemolytic buffer), incubated for 2 minutes at 22°C and the remaining granulocytes pelleted by centrifugation.

Alternatively, where the principal contaminating cells are red cells, it may be possible to partially purify granulocytes from an apheresis product by simply allowing said apheresis product to settle by gravity for about an hour, during which time the red cells will settle to the bottom of the bag containing the apheresis product. The granulocytes can then be expressed carefully from the bag, leaving the red cells behind.

Granulocytes may also be partially purified from an apheresis product by immunoselection, for example, using monoclonal or polyclonal antibodies, or fragments thereof, specific for antigens expressed on the surface membrane of granulocytes. Numerous antibodies have been described in the literature which are useful for purifying granulocytes (*see Leucocyte Typing IV*, W. Knapp (ed.), Oxford: Oxford UP, 1989) and include antibodies belonging to such clusters of differentiation as CD15, CD66, CD67, etc. Not only can antibodies which bind predominantly to granulocytes be utilized, but also antibodies which fail to bind granulocytes while binding to most other leukocytes; that is to say, granulocytes can be purified by either positive or negative immunoselection. Further, antibodies may be employed as mixtures, or cocktails, to achieve the desired degree of specificity and sensitivity.

For immunoselection, antibodies may be immobilized on a solid support, such as beads, fibers, plastic dishes, etc., or may be labeled, for example, with a fluorophore, and used in solution to select for the desired cells. Methods of immunoselection suitable for use within the present invention include column chromatography (Berenson et al., WO 87/04628, published August 13, 1987; Berenson et al., in *Recent Adv. Leuk. Lymphoma*, NY: Alan R. Liss, pp. 527-533, 1987; Berenson et al., in *Prog. Bone Marrow Transplant.*, NY: Alan R. Liss, pp. 423-428, 1987), panning (Wysocki and Sato, *Proc. Natl. Acad. Sci. (USA) 75*(6):2844-2848, 1978) and fluorescence activated cell sorting.

If partial purification of granulocytes is undertaken, it is generally desired to enrich the proportion of granulocytes about 2-fold. Typically, a granulocyte suspension after partial purification by one or more of the above methods will contain at least about 90% granulocytes (in terms of cell number),

more often 95% granulocytes. It is also preferred that the hematocrit of the granulocyte suspension be less than 30%, preferably less then 20%, and most preferably about 15% or less. However, it should be noted that crude granulocytes, such as granulocytes obtained by apheresis in the presence of HES, can generally be cryopreserved by the methods of this invention as well as more purified granulocytes.

Typing of Granulocytes

Using the methods of this invention, both autologous (self-derived) and allogeneic (donor-derived) granulocytes may be collected, cryopreserved, and used to treat patients exhibiting fever and infection, or patients at risk thereof. If allogeneic granulocytes are contemplated for prophylaxis or treatment, it is generally desirable to utilize granulocytes from an identical twin (syngeneic) or from an HLA-matched, preferably related, donor.

Methods of HLA typing cells are well-known in the art and are described, for example, in Zachary and Braun (Eds.), AACHT Laboratory Manual (1981); Terasaki, *Am. J. Clin. Pathol. 69*(2): 103, 1978; and Drew et al., *Tissue Antigens 12*(2): 75, 1978. In the most widely used method, the dye-exclusion microcytotoxicity assay, peripheral blood lymphocytes are isolated by density gradient centrifugation in Ficoll (1.077 g/L), and dispensed into wells in a Terasaki tray in the presence of HLA typing sera and complement. After incubation, a vital dye is added and each well is examined microscopically to determine the extent of cell lysis. Cells which express a given HLA antigen will be lysed by the serum which recognizes that antigen, enabling one to determine the HLA type of the individual from whom the cells were derived. Typically, HLA typing is performed using alloantisera derived from multiparous women or from individuals who have been repeatedly transfused. Monoclonal antibodies can also be used, however (Morris, *Transplant 36*:719, 1983). Although dye-exclusion microcytotoxicity is the method typically used for HLA typing, other methods can also be used, such as ELISA.

HLA matching is especially important if the patient has already been sensitized (alloimmunized) by prior transfusion of blood products. If the patient has not been alloimmunized, HLA matching is less critical.

Generally, it is desirable to use HLA-matched granulocytes from a related donor, if allogeneic granulocyte transfusions are contemplated. Alternatively, HLA-matched, unrelated-donor granulocytes may be employed. On occasion, even HLA-mismatched granulocytes may be employed.

Granulocytes also express other, non-HLA encoded, polymorphic antigens to which a patient can become sensitized. Several assays have been described for the detection of such granulocyte-specific (leukoagglutinating) antibodies (reviewed in Schiffer, *Cancer Treatment Rep. 67*:113, 1983). In previously alloimmunized patients, it may be important to use a granulocyte antigen-matched donor.

In addition, it is highly desirable that donor granulocytes be derived from a donor who has not been exposed to any blood-borne viruses and who is negative, for example, for hepatitis B surface antigen (HBsAg), and negative for antibodies to CMV, HIV 1 and HIV 2.

Cryopreservation of Granulocytes

According to the methods of the present invention, granulocytes may be prepared for cryopreservation by suspending them in a cryopreservation (freezing) medium. Within one embodiment of the invention, the cryopreservation medium is comprised of a penetrating cryoprotectant, an anticoagulant, and/or a nuclease. Within further embodiments, the cryopreservation medium may further comprise a physiologically acceptable medium, and/or a source of protein, such as human serum albumin (HSA). Preferably, each of the components of the cryopreservation medium (*e.g.*, the source of protein) is selected such that it is also physiologically acceptable.

Representative examples of physiologically acceptable media suitable for use within the present invention include phosphate buffered saline (PBS), medium 199, RPMI, and DMEM, among others, with medium 199 and PBS being particularly preferred. Such media may be readily obtained from commercial suppliers (*e.g.*, GIBCO/BRL, New York or BioWhittaker, Walkersville, MD) or prepared in accordance with standard formulations (see ATCC: catalogue of cell lines and hybridomas, "Media Formulations," 7 ed., 1991). PBS consists of KH_2PO_4 (144 mg/L), NaCl (9000 mg/L), and Na_2HPO_4 (795 mg/L).

Sources of protein suitable for use with the cyropreservation media may be obtained from humans or other animals, such as bovine, and includes for example, blood serum or plasma or a protein derived therefrom, such as albumin. Human serum albumin (HSA) at a final concentration between about 2%-8% (w/v), more often at about 4%, is particularly preferred.

The penetrating cryoprotectant is typically DMSO, and may be added to a final concentration between approximately 5% (v/v) and 10%, more often between about 7.5% and 10%, and most often about 7.5%.

Among anticoagulants which may be used are CPDA, acid citrate dextrose (ACD-A or ACD-B), EDTA and heparin, with the latter especially preferred. Either preservative-free heparin or heparin preserved in benzyl alcohol may be used, the former being preferred. Heparin may also be added to a concentration between approximately 50 and 150 units/mL, more often between about 75 and 120 units/mL, and preferably about 100 units/mL.

Among nucleases which may be used are ribonuclease (RNase) and DNase, with DNase being preferred. Within one embodiment, DNase may be added to a concentration between approximately 50 and 150 units/mL, more often between about 75 and 120 units/mL, and preferably about 100 units/mL. The number of units of DNase activity per mg of protein will vary. In general, however, 100 units will correspond to between about 10 and 100 μg of protein, more often about 20-50 μg.

Multiple sources of medium, DMSO, heparin, and DNase are known in the art. Preferred sources include Tera Pharmaceuticals (Salt Lake City, Utah) for DMSO, Sigma (St. Louis, MO) for heparin, Genentech for DNase, and BioWhittaker for medium.

Either type I or type II DNase is acceptable in the methods and compositions described herein. DNase is typically available from commercial sources partially purified from bovine pancreas. Bacterial sources of DNase are also known (see, for example, Sherry et al., *Exp. Biol. Med. 68*:184, 1948). Recombinant human DNase (as described in EP449968, which is herein incorporated by reference) is also available (Genentech, So. San Francisco, CA) and may also be readily utilized within the methods of this invention. Briefly, the DNase is typically made up to a concentration of 10,000 units/mL (or about 4 mg/mL in a buffer such as 140 mM NaCl/1 mM $CaCl_2$ and stored frozen until needed. Preferably, the DNase is thawed immediately before it is added to the freezing medium, usually within about 15 minutes of adding to the freezing medium and, preferably, within about 5 minutes of addition. Alternatively, DNase may be provided as a lyophilized composition which is reconstituted in a suitable buffer or in water prior to use.

Within one embodiment of the invention, the cryopreservation medium may further comprise a granulocyte mobilization agent (*e.g.*, G-CSF, GM-CSF, SCF, IL-1, IL-3 or PIXYKINE). The granulocyte mobilization agent

may be added to a final concentration of about 1 to 1000 ng/mL, and most preferably, to a final concentration of about 10 to 100 ng/mL.

The cryopreservation medium may be prepared as a working strength (1X) solution of HSA, DMSO, heparin and DNase in medium 199 or PBS and chilled prior to addition to the cells. Alternatively, equal parts of a 2X solution of HSA (2X = 8%)/1X heparin/1X DNase in TC199 or PBS and a solution of 2X (2X = 15%) DMSO/1X heparin/1X DNase in TC199 or PBS can be added to the cells.

In yet another alternative embodiment, the cryopreservation medium is comprised of DMSO, heparin, and DNase, and may be prepared as a 1X solution, or each component of the cryopreservation medium may be packaged separately and added to the cells one component at a time. Where the latter embodiment is employed, it is preferred to add the components in the following order: heparin, followed by DNase, followed by DMSO. Heparin is generally provided as a 2000 U/mL stock solution and DNase as a 4 mg/mL stock solution.

For the purposes of this invention, the cell density at which cells are frozen may vary between about 1×10^6 and 700×10^6 cells/mL. Generally, it is preferred that the cell density be between about 100×10^6 and 500×10^6 cells/mL, more often between about 250 and 500×10^6 cells/mL. The cell density can be conveniently determined by counting an aliquot of cells using a hemacytometer and a light microscope, or an electronic impedance counter, such as a Coulter counter (Coulter, Hialeah, FL). Knowing the number of cells in a given volume, the density can then be adjusted by dilution or, if the cell suspension is too dilute, the cells can be pelleted by centrifugation and resuspended in a smaller volume.

Granulocytes suspended to the desired concentration in cryopreservation medium may be divided into a plurality of aliquots, each aliquot approximating a therapeutically or prophylactically effective dose of granulocytes. The aliquots are then frozen to a temperature between about -20°C and -196°C, preferably at a controlled rate of freezing, for example, as described below. Each aliquot will typically contain about 500 million to about one billion cells in a volume of about 10-60 mL, more often about 20-60 mL, and typically about 40-50 mL.

It will be appreciated that the number and concentration of granulocytes in an aliquot may be adjusted at the discretion of a patient's health care provider. Similarly, the dose of granulocytes administered to a patient in

need thereof can be varied, again at the discretion of the patient's health care provider, by thawing and administration of multiple aliquots (should a larger dose be desired) or by administration of a fraction of an aliquot (should a smaller dose be desired).

Granulocytes suspended to the desired concentration in cryopreservation medium may be dump-frozen, *i.e.*, without controlling the freezing rate, or frozen at a controlled rate. A number of devices are commercially available for controlled-rate freezing of biological materials, for example, from Cryomed (New Baltimore, MD). Typically, the granulocyte suspension is cooled to 4°C, then to -4°C at -1°/min, then to -40°C at -25°/min, then to -12°C at -15°/min, then to -40°C at -1°/min, and finally to -90°C at -10°/min. Upon completion of freezing, cells are removed to the vapor phase of liquid N_2. The temperature at which granulocytes are stored frozen is generally as low as possible, given the freezing equipment available. If liquid N_2 storage is not possible, storage in a mechanical freezer is possible. In general, controlled-rate freezing is preferable to dump freezing, although dump freezing is acceptable if a programmable freezing unit is not available.

It is generally preferred that granulocytes be transferred to cryopreservation medium as soon as possible after collection and purification, typically within one hour. Ideally, the entire series of collection steps, from apheresis to commencement of freezing, should take no longer than about 1-2 hours. If delays are encountered at any step in the collection process, it is preferable to store the granulocytes at about 22°C (room temperature) until processing can resume. If the granulocytes must be held at some point in the collection process, they are best held in medium 199 or PBS containing about 4% (w/v) HSA, with or without DNase and heparin.

In one embodiment of the instant invention, granulocytes are harvested by apheresis of a G-CSF mobilized donor or patient in the presence of hydroxyethyl starch and resuspended at a concentration of approximately 500×10^6 cells/mL in a freezing medium consisting of 7.5% DMSO, 100 units/mL heparin, 100 units/mL (40 μg/mL) DNase, with or without 4% (w/v) HSA in PBS; and frozen in multiple aliquots at a controlled rate to a temperature between about -80°C and -196°C. When granulocyte collection and cryopreservation are carried out as described, the recovery (ratio of cells thawed to cells frozen) after thawing is between approximately 80 and 90%, cell viability is between approximately 80% and 90%. In addition, the granulocytes retain at least about 30% of their biological activity relative to fresh normal

donor granulocytes as determined in an intracellular killing assay, more often about 50%-75% of their biological activity, or alternatively, retain about 10%, more often 15% to 20% of their activity relative to fresh donor granulocytes in a respiratory burst activity assay.

Irradiation of Granulocytes

Within certain embodiments of the invention, granulocytes may be irradiated, for example, from an electron beam source such as a linear accelerator, prior to cryopreservation. Typically, irradiation with about 1500-4000 rads, typically about 2500 rads, usually at a rate of about 150-250 rads/min, is sufficient. During irradiation, the granulocytes are suspended in a physiologically acceptable medium, such as medium 199 or PBS, containing about 8% (w/v) HSA, with or without DNase and heparin (each at about 100 units/mL). Alternatively, granulocytes may be irradiated using a Gamma Cell 1000 (Nordion Int'l, Ontario, Canada). In this embodiment, irradiation with about 25 Gy for 2-5 minutes is usually sufficient. During irradiation, the granulocytes are suspended in the transfer pack in which they were collected from the apheresis machine. No additional medium or protein is added.

Irradiation is typically performed to eliminate mature lymphocytes, which are highly radiosensitive, and which mediate graft-vs-host disease. Hence, generally only allogenic (donor) granulocytes are irradiated. However, it may be advantageous in certain instances to irradiate autologous (patient) granulocytes, for example, to kill contaminating tumor cells present in the granulocyte fraction. The amount of radiation necessary to kill contaminating tumor cells will vary, depending on the type of tumor, some tumor cells being more radiosensitive than others, and can be determined empirically. Generally, the highest dose of radiation which can be tolerated by granulocytes without substantially reducing their biological activity is utilized.

Assays of Granulocyte Biological Activity

Post-thaw granulocyte viability may be conveniently determined by any of several methods which are known in the art, of which the most common is dye exclusion. For example, cells may be stained with a vital dye, such as Trypan blue or Janus green B, and the number of viable cells determined by counting the cells under a light microscope (Zaroulis & Leiderman, *Cryobiol. 17*:311, 196). Alternatively, the cells may be stained with a fluorescent vital dye, such as propidium iodide, and examined by fluorescence microscopy or

fluorescence activated cell sorting. Live cells exclude the dye, while dead cells stain with the dye. Generally, it is desired that viability as assayed by dye exclusion be greater than about 80%, and preferably, greater than about 90%.

While viability is important for cells which are to be transfused into a patient, it is not the only determinant of product quality which must be considered. In particular, granulocytes may appear viable (for example, by propidium iodide staining) and may have a normal light scatter profile by flow cytometry, but they may lack biological activity. Therefore, it is generally desirable that the granulocyte concentrates prepared by the methods of this invention also retain a substantial fraction of their biological activity, as gauged by locomotion, dye reduction capability, their response to chemotactic stimuli, their bactericidal capability, their phagocytic capability, etc. These functions may be assayed as described below.

Chemotaxis can be conveniently assayed in a Boyden chamber, using zymosan-activated serum, HBSS, or the peptide F-Met-Leu-Phe as chemoattractant (Hill et al., *Crybiol. 18*:533, 1981). Alternatively, migration under agarose can be performed as described in Nelson et al., Leukocyte Chemotaxis, Gallen & Quie (eds.), N.Y., Raven, p. 25, 1978, or Nelson et al., *Immunol. 115*:1650, 1975.

The ability of granulocytes to phagocytose opsonized particles can be determined by measuring the cells' chemiluminescence (Grebner et al., *J. Lab. Clin. Med. 89*:153-159, 1977; Nelson et al., *Infect. Immun. 14*:129-134, 1976). Typically, granulocytes are incubated with a suspension of opsonized yeast or zymosan-coated bacteria in a medium containing luminol and the light emission from the granulocytes quantified in a liquid scintillation counter, using the tritium channel (Ziprin, *Infec. Immun. 19*:889-892, 1978), or in a luminometer (Caspar et al. op. cit.).

Luminol enhanced chemiluminesence can also be used as a measure of respiratory burst activity in granulocytes frozen and thawed according to the methods of this invention. Briefly, about $1x10^6$ cells at a concentration of $10x10^6$/mL are added to a polystyrene chemiluminescence cuvette and the cuvette is filled with RPMI containing 10 mM HEPES and 15 μg/mL HSA. The assay is initiated by the addition of 10 μM luminol and 1 μM phorbol myristate acetate (PMA). Luminescence readings are taken at intervals over a period of 2 hours using a Monolight 2001 Luminometer (Analytical Luminescence Labs, San Diego, CA), set on integration mode.

Phagocytic activity can also be assayed by incubating granulocytes with zymosan-coated latex particles or opsonized yeast particles or bacteria, such as *Staphylococcus aureus* or *E. coli*, in medium at 37°C. At various intervals, aliquots are withdrawn, treated with 0.2 nM N-ethyl maleimide to prevent further phagocytosis, cytocentrifuged onto slides, and examined microscopically for the presence of engulfed bacteria (Baldwin et al., *Proc. Natl. Acad. Sci. (USA) 85*:2763, 1988; Schiffer et al., *Transfusion 15*:473, 1975; Takahashi et al., *Crybiol. 22*:215, 1985; Caspar et al., op. cit.).

Intracellular killing can be assessed by incubating granulocytes with *S. aureus* or *E. coli* bacteria for a period of time, in the presence or absence of a cytokine such as G-CSF, withdrawing aliquots at intervals, lysing the cells, and plating the aliquots on a medium capable of supporting bacterial growth, such as trypticase soy agar. Bacterial colonies are counted at each time interval and compared with the number of colonies which could be grown out at time zero (Baldwin et al., *op. cit.*; Schiffer et al., *op. cit.*; Takahashi, *op. cit.*). Such an assay measures both the ability of granulocytes to phagocytose bacteria and to kill them once they have been ingested.

Another assay of granulocyte function is antibody-dependent cell mediated cytotoxicity (ADCC). Typically, granulocytes are suspended in medium containing GM-CSF and incubated with a radiolabeled (usually ^{51}Cr) target cell line in the presence of an antibody cytotoxic for the target cells. Chromium release is measured after a three-hour incubation and compared to the amount of release from target cells incubated without granulocytes (Baldwin et al., *op. cit.*).

Locomotion can be assessed by observing the formation of pseudopodia microscopically. Dye reduction can be assayed using nitroblue tetrazolium (NBT) essentially as described in Hill (*op. cit.*). Various tinctorial properties of granulocytes can also be assayed. For example, toluidine blue, Wright-Giemsa, Feulgen, and periodic acid-Schiff (PAS) stains can be used to assess morphologic changes in post-thaw granulocytes (Malinin, *Cryobiol. 9*:123, 1972).

Granulocyte adherence can be assayed by plating $2x10^6$ cells suspended in 1 mL RPMI containing 10% human plasma and 10 μ/mL heparin on to PERMANOX dual chamber slides (Nunc, Naperville, IL). The slides are incubated at 37°C in a humidified atmosphere containing 5% CO_2 and evaluated at various intervals over about a 3 hour time period. Briefly, about 900 μg of the cell suspension is removed from the slide and counted, for example, in an

electrical impedance counter, to assess how many cells are in suspension. Adherent cells, *i.e.*, those cells remaining on the slide, are stained with a fluorescent vital dye and examined microscopically.

Granulocytes prepared by the methods of this invention are said to retain a substantial fraction of their biological activity if their post-thaw activity is at least about 30% of the activity of fresh (unfrozen) granulocytes (from the same or a different donor) in an intracellular killing assay. Ideally, their post-thaw activity will more often be at least about 50% of the activity of fresh granulocytes on an intracellular killing assay.

When granulocytes prepared by the method of this invention are assayed for biological activity using chemiluminescence to quantify their respiratory burst activity (*e.g.*, in a "respiratory burst activity assay"), they are said to be biologically active if they retain at least 10% of the activity of fresh (unfrozen granulocytes), more often at least about 15%.

Within the context of the present invention, pre-freeze activity is determined after purification, if any, but before resuspension in freezing medium. Post-thaw activity is determined after thawing and dilution, preferably at a fixed time point after thawing and dilution, and generally after the cells have been washed to reduce or eliminate DMSO.

While the best measure of retention of biological activity may be to compare pre-freeze and post-thaw granulocytes from the same patient or donor, it is often more practical to compare post-thaw granulocytes to freshly collected granulocytes from the same or a different donor. Since granulocyte activity may vary among individuals and between collections from a single individual, post-thaw activity may be either more or less when compared to the activity of freshly drawn granulocytes than when compared to the activity of pre-freeze granulocytes from the same patient or donor.

The actual values obtained in the above-discussed functional assays will vary from day to day and from donor (or patient) to donor (or patient). Hence, the biological activity of granulocytes prepared by the methods of this invention is expressed as a percentage of the control, rather than as an absolute number, and the control is normalized to 100%. Likewise, it will be appreciated by those skilled in the art that assay conditions and other factors, such as the health of the donor (or patient), other medications the donor (or patient) may be receiving, and the number of prior leukaphereses the donor (or patient) has undergone, may influence the results of the above-mentioned functional assays and hence, the desired ratios of pre-freeze and post-thaw

activity may not always fall within the ranges specified, although they will tend to do so if averaged over a large enough number of patient (or donor) samples.

Methods for Thawing Granulocytes

Granulocytes frozen according to the methods of this invention may be stored for at least about 12 weeks. In general, the lower the freezing temperature, the longer storage is possible without deterioration in biological activity or viability. However, practical considerations, such as the type of freezer available, may limit the length of storage.

In one embodiment of this invention, the granulocyte concentrate is thawed immediately before use. In general, rapid thawing is preferred and can be achieved, for example, by placing the vessel in which the cells were frozen in a 37°C water bath. As soon as the cells are thawed, they are diluted by the dropwise addition of an equal volume of PBS/heparin (100 U/ml)/DNase (100 U/ml) (dilution medium) pre-warmed to 37°C. After mixing, additional dilution medium is added gradually until the cell suspension has been diluted approximately 10-fold. Depending on the concentration at which the cells were frozen initially, they may be diluted more or less than 10-fold upon thawing. The cells are then pelleted by centrifugation, the supernatant discarded and the pellet resuspended in a medium suitable for infusion or further testing, such as PBS/heparin (100 U/mL)/DNase (100 U/mL).

If desired, an aliquot of the thawed cells may be removed for functional assay, flow cytometry analysis, sterility testing or such other assays as are deemed appropriate.

Indications for Granulocyte Transfusion

Granulocyte transfusions may be indicated in patients who are granulocytopenic due to myelotoxic chemotherapy, radiation therapy, immunosuppressive therapy, accidental radiation exposure, immunosuppression due to an infectious agent(s), malignancy, post-operative or post-traumatic blood loss, or idiopathic and hereditary defects, such as aplastic anemia. A patient may be considered granulocytopenic if his absolute neutrophil count (ANC) is less than about 1000 cells/uL, more often less than about 500 cells/uL, and most often less than about 100 cells/uL.

Granulocyte transfusions may also be indicated in patients whose granulocytes are dysfunctional, although their numbers may be normal or near

normal. Chronic granulomatous disease is one example of granulocyte dysfunction in which granulocyte transfusions may be indicated.

Granulocyte transfusions may also be indicated in patients who are predisposed to fever or infection for any of a variety of reasons in addition to those mentioned above, such as damage to the anatomical barrier (for example, due to radiation injury or thermal or chemical burning) and obstructive phenomena (see Schimpff, in *Principles and Practice of Infectious Disease*, Mandell et al. (eds.), 3d ed., NY:Churchill Livingstone, p. 2258, 1990).

A granulocyte transfusion is considered to be therapeutic if it is given to a patient with fever and/or infection. Fever is defined as a persistent daily elevation in oral temperature to 38°C (100.2°F) or higher. Infection is defined as the multiplication of a microbe, in or on the patient, which microbe is a pathogen in a non-sterile site. Infection may be culture-proven or clinically obvious. Signs and symptoms of infection may include fever, chills, cough, dyspnea, headache, radiographic changes, neurologic signs, loss of appetite, decreased awareness, and/or seroconversion. Infection may occur in virtually any organ or tissue, including blood (sepsis or septicemia), periodontium, oropharynx, sinus, lung, gastrointestinal tract, genitourinary tract, and skin. Infection may be caused by any of a variety of microbes, including for example viruses, gram-negative bacteria, gram-positive bacteria, protozoa, and fungi.

Since may of the signs and symptoms of infection in a patient are due to an inflammatory response, which is impaired in a granulocytopenic or granulocyte-dysfunctional patient, such a patient may not show any or all of the usual symptoms of infection, such as a purulent discharge, an elevated white cell count, an elevated erythrocyte sedimentation rate (ESR), and a rise in acute phase reactants, such as C-reactive protein (Sickles et al., *Arch. Intern. Med. 135*:715, 1975). Consequently, the diagnosis of infection in a granulocytopenic or granulocyte-dysfunctional patient must often be made clinically. An expanded discussion of fever and infection can be found in Dinarello and Wolfe, in *Principles and Practice of Infectious Disease*, *op. cit.*, p. 462, and in Meyers, *ibid.*, p. 2291.

Fever is considered to be reduced or eliminated (fever lysis) if the patient's oral temperature is persistently less than 38°C. Infection is considered to be reduced or eliminated if a culture-positive patient becomes culture-negative; or if there is improvement in clinical parameters, such as appetite, alertness, average height of fever, or stabilization of a downhill course; or if

there is improvement in other signs and symptoms, such as radiographic improvement, resolution of cough, dyspnea, etc.

Generally, it is desirable to commence granulocyte transfusion therapy as soon as fever is observed or infection is suspected. Transfusions are continued until the infectious episode resolves or until there is bone marrow recovery, as evidenced by a self-sustaining ANC of at least about 100 cells/uL. In most instances, granulocyte transfusion therapy will be given along with antibiotic (or antifungal or antiviral or antiprotozoal) therapy. Generally, antibiotic therapy with one or more broad-spectrum antibiotics will be commenced at the first sign of infection, if the patient is not already receiving antibiotics prophylactically. Among antibiotics commonly used to treat or prevent infections in granulocytopenic patients are quinolones (*e.g.*, ciprofloxacin), vancomycin, sulfa drugs, beta-lactams, and aminoglycosides, among others. Antifungal agents include amphotericin B, trimethoprimsulfa methoxazole, fluconazole, etc. Antiviral agents include ganciclovir, acyclovir, and ribavarin. Antiprotozoal agents include metronidazole.

A granulocyte transfusion is considered to be prophylactic if it is administered to a patient who is at risk of fever or infection. Typically, a patient is considered to be "at risk" from the onset of granulocytopenia until recovery of a normal granulocyte count. This period of risk may be defined differently in different patient groups, but generally corresponds to the period during which the patient's ANC is less than about 100 cells/uL. During the "at risk" period, patients may also receive prophylactic antibiotics (or antifungals, antivirals, or antiprotozoals) concurrently or intercurrently with granulocyte transfusions.

Methods of Granulocyte Transfusion

Typically, granulocytes are frozen in aliquots, each aliquot containing a therapeutically or prophylactically effective dose of cells. For example, a typical aliquot might contain approximately 10^9 to 10^{10} granulocytes, an amount sufficient for therapy of fever or infection in an average, 70 Kg male patient, or 10^7 to 10^8 granulocytes, for prophylaxis of fever or infection in an average 70 Kg male patient. In general, this amount of granulocytes will be sufficient to effect an increment of 100 cells/μL in the patient's ANC. It will be appreciated, however, that the exact dose required may vary from patient to patient and can be adjusted, as necessary, by altering the number of aliquots the patient receives at one time or by altering the schedule of administration.

Generally, it is desirable to use HLA-matched granulocytes from a related donor, especially if the patient has been alloimmunized by prior transfusion of blood products (such as platelet concentrates), if allogeneic cells are to be used. Likewise, in an allogeneic setting it is desirable to use granulocytes from a cytomegalovirus (CMV)-seronegative/HIV-seronegative/HBSAg-negative donor. Since CMV-seronegative donors tend to be rare, CMV-seropositive donors are sometimes used for CMV-seropositive patients.

The thawed granulocyte concentrate may be infused into the patient intravenously through a needle and syringe, a standard blood administration set, or through an indwelling catheter. The concentrate may be infused at a rate between 1×10^{10} and 10×10^{10} cells/hour to minimize adverse reactions in the recipient. Alternatively, the cells may be infused as a single bolus.

Various medications may be co-administered during the transfusion, such as analgesics (*e.g.*, acetaminophen), antihistamines, sedatives, steroids, etc.

The optimum dose of granulocytes and schedule of administration is determined empirically in each patient group. Generally, granulocyte transfusions are given on a schedule which enables the ANC to be maintained at or above 100 cells/uL. This can usually be accomplished by daily transfusions of 10^8-10^{10} granulocytes. However, count increments may be difficult to interpret, since granulocytes will migrate to sites of infection, where they may be effective without causing an increase in the circulating ANC.

The dose and schedule of granulocyte transfusions can be adjusted independently or concomitantly, if necessary, to achieve any desired absolute neutrophil count (ANC). Cytokines, such as G-CSF, GM-CSF, IL-3, SCF and IL-1 may be given in conjunction with the granulocyte concentrate.

Occasionally, it may be necessary or desirable to transfuse a patient at more frequent than daily intervals, such as about every 12 hours, or at less frequent intervals, such as about every 36 to 72 hours.

Longer intervals between transfusions may be possible, especially when transfusing mobilized granulocytes, which include committed progenitor cells. These cells can temporarily "engraft" the patient, leading to count increments which may be sustained for several days. Typically, a therapeutic dose of granulocytes will be larger than a prophylactic dose in a patient of the same weight. However, there may be considerable variability among patients

and hence it will be appreciated by one skilled in the art that the dose and schedule of administration will be determined empirically in each patient population.

Additional Embodiments of the Invention

As noted above, the present invention provides methods for the collection and cryopreservation of granulocytes, particularly human granulocytes, for the prevention and treatment of fever and infection by granulocyte transfusion. In one embodiment thereof, a granulocyte fraction is obtained from the peripheral blood of a mobilized patient or donor. This fraction is then resuspended in a freezing (cryopreservation) medium which comprises a penetrating cryoprotectant, an anticoagulant and/or a nuclease, and frozen to a temperature between about -20°C and -196°C, preferably between about -80°C and -90°C, and preferably at a controlled rate of freezing.

In another embodiment of the invention, the granulocyte fraction is divided, after resuspension in freezing medium, into a plurality of aliquots, each aliquot approximating a therapeutically or prophylactically effective dose of granulocytes ($1x10^9$ - $25x10^9$ cells). Each aliquot is then frozen to a temperature between about -20°C and -196°C, preferably at a controlled rate of freezing. Where the granulocytes are contemplated for therapeutic use, that is, in a patient with documented fever or presumptive or documented infection, each aliquot will typically comprise about 10^7 - 10^{10} granulocytes in a volume of about 10-60 mL, typically about 40-50 mL, prior to thawing and dilution. Where the granulocytes are contemplated for prophylactic use, that is, in a patient at risk for fever or infection due to a concurrent or intercurrent condition, each aliquot will typically comprise about 10^6 - 10^9 granulocytes in a volume of about 10-60 mL, typically about 40-50 mL, prior to thawing and dilution. To achieve a larger dose, multiple aliquots may be transfused. To achieve a smaller dose, a fraction of an aliquot may be administered.

The instant invention also provides compositions for the cryopreservation of granulocytes, which compositions are comprised of a penetrating cryoprotectant, an anticoagulant, and a nuclease. Said compositions are generally provided in lyophilized form or as sterile or aseptic solutions. Alternatively, they may be provided as non-sterile solutions which can be sterilized prior to use, for example, by membrane filtration. The cryopreservation compositions of the instant invention may be provided as a single solution or as two or more solutions which are combined during use.

Alternatively, they may be provided as one or more solutions and one or more lyophilized reagents which are combined during use. Similarly, the compositions may be provided at working strength (1X concentration) or higher.

According to one embodiment of the invention, a kit is provided comprising a primary vessel having a volume of about 600 mL, said vessel being interconnected via sterile pathways to between about 5 and about 15, typically about 10, empty satellite vessels, each satellite vessel having a volume between about 30 mL and about 60 mL. The primary and satellite vessels may be flexible or rigid, most often flexible, and may be fabricated from any of a variety of materials, among which the most common are polyvinyl chloride (PVC), ethyl vinyl acetate (EVA), and TEFLON. Also provided in the kit are sources of anticoagulant, penetrating cryoprotectant, and nuclease. Preferably, the anticoagulant is heparin; the penetrating cryoprotectant is DMSO; and the nuclease is human recombinant DNase. These components may be provided as liquid or lyophilized compositions and are preferably sterile or aseptic.

Granulocytes are collected by apheresis in the presence of a red cell sedimenting agent, such as hetastarch. Typically, the granulocytapheresis product is collected in a volume of about 250 mL - 750 mL, more often about 300 mL - 600 mL and is comprised of about 100 billion cells, of which about 70% are cells of the myeloid lineage. The other 30% of cells may be mononuclear cells, red blood cells, and/or platelets. The collection bag containing the granulocytapheresis product is allowed to sit for approximately one hour in order that any contaminating red cells will settle to the bottom of the bag. The granulocytes are then expressed from the collection bag, under aseptic conditions, into the primary vessel provided with the kit. Alternatively, the granulocytes may be transferred immediately from the collection bag to the primary vessel and the red cells allowed to sediment to the bottom thereof. If the hematocrit of the granulocytapheresis product is sufficiently low (less than about 25%), it may not be necessary to sediment the red cells. Cryoprotectant, anticoagulant, and nuclease are added aseptically to the primary vessel in amounts sufficient to achieve the desired final concentrations of each, in accordance with the methods of the instant invention, and the contents of the bag are mixed. The contents of the primary vessel are then expressed into each of the satellite vessels in approximately equal volumes. Once filled, the satellites are disconnected from the primary vessel and frozen to a temperature between about -20°C and -196°C, in accordance with the methods of this invention.

The instant invention also provides compositions for the prevention and treatment of fever and infection in patients at risk therefor. Briefly, these compositions include a prophylactically or therapeutically effective dose of thawed granulocytes suspended in a medium which is acceptable for transfusion, wherein the granulocytes retain at least about 30% of their biological activity relative to fresh donor granulocytes. Typically, a prophylactically effective dose will comprise about 10^6 - 10^9 granulocytes/Kg, usually about 10^7 - 10^8 granulocytes/Kg. A therapeutically effective dose will comprise about 10^7 - 10^{10} granulocytes/Kg, usually about 10^8 - 10^9 granulocytes/Kg. Also contemplated are compositions of frozen granulocytes suspended in the cryopreservation medium of the instant invention.

When granulocytes are collected and cryopreserved according to the methods of the present invention, their post-thaw recovery (*i.e.*, the number of cells which survive freezing and thawing) has been found to be at least about 75%, more often at least about 80%. In addition, the recovered cells are substantially viable (greater than about 80%, more often greater than about 85%, preferably at least about 90%), as assessed by a dye exclusion assay, and retain at least about 30% of their biological activity, more often, about 50%-75% of their biological activity, as assessed by an intracellular killing assay relative to fresh donor granulocytes, or at least about 10% (and more often at least about 15% to 20%) of their respiratory burst activity, as assessed by a chemiluminescence assay relative to fresh granulocytes..

Accordingly, there are a number of patient groups which may benefit from transfusion of such granulocyte concentrates due to the risk (or presence) of fever or infection. These include bone marrow transplant patients (both autologous, syngeneic and allogeneic); patients on high dose, myelotoxic chemotherapeutic regimens; patients on immunosuppressive regimens; patients with sepsis (especially neonates); burn patients; patients receiving radiation therapy; persons inadvertently exposed to myelotoxic amounts of radiation; patients with immune deficiency disorders; persons who have experienced blood loss, for example, as a result of trauma or surgery; and patients with idiopathic or hereditary defects in granulocyte number or function. In general, any patient who is at risk of fever or infection due to reduced number or function of granulocytes, regardless of cause, is a candidate for transfusion of granulocyte concentrates. The number of granulocytes which is considered to be a reduced number will vary depending on the clinical settling, but is generally <1000 cells/uL, more often <500 cells/uL, and most often <100 cells/uL.

Although allogeneic (donor) granulocyte transfusions may be acceptable in certain clinical settings (and indeed, are the only alternative for patients with hereditary defects in granulocyte number or function), autologous (self) or syngeneic (identical twin) transfusions are often preferred, as there are fewer potential side-effects associated therewith. Previously, the large number of granulocytes needed, coupled with the inability to store them for any appreciable length of time necessitated that most granulocyte transfusions were allogeneic. However, the instant invention makes it practicable to collect large volumes of granulocytes over a period of days or weeks from a patient (for example, prior to high dose chemotherapy or bone marrow or peripheral blood stem cell transplantation), aliquot them, store them for prolonged periods of time, and thaw aliquots for re-infusion into the patient on an as-needed basis.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

EXAMPLE 1

COLLECTION AND CRYOPRESERVATION OF GRANULOCYTES FROM NORMAL DONORS. EFFECT OF PROCESSING STEPS, DNASE, AND HEPARIN ON GRANULOCYTE RECOVERY

A. Collection and Processing of Granulocytes

7.5 mL of heparinized blood, is collected by venipuncture of normal donors, and diluted 1:1 with PBS (Ca^{2+}, Mg^{2+}-free) and layered over an equal volume (15 mL) of Ficoll (1.077 g/L) in a 50 mL centrifuge tube. After centrifugation for 30 minutes at 1700 rpm, the plasma and mononuclear cell layer is aspirated off and discarded, leaving a layer of granulocytes above the red cells.

The resultant granulocyte fraction is partially purified by one of three methods in order to reduce or remove contaminating red cells. In the first method (hereinafter dextran sedimentation), 25 mL of dextran (Sigma D-4751; 3% (w/v) in 0.9% NaCl, filter sterilized) are added to the granulocyte fraction, and the resultant mixture is allowed to settle for one hour at room temperature (22°C-25°C). After settling, the supernatant is withdrawn and the cells suspended therein pelleted by centrifugation, and washed once in 40 mL of PBS. The supernatant from the wash is then withdrawn and discarded. The remaining cell pellet is frozen as described in (B) below.

In the second method (hereinafter hemolysis), 30 mL of ammonium chloride (8.3 g/L NH_4Cl, 1.0 g/L $NaHCO_3$, 0.4 g/L EDTA) are added to 10 mL of packed granulocytes. The cells are resuspended and incubated for two minutes at 22°C to hemolyse the red cells, after which the remaining granulocytes are pelleted by centrifugation, washed once with PBS, and frozen as described below in subparagraph (B).

In the third method (starch sedimentation), hetastarch (HESPAN; 6% in 0.9% NaCl) is added to the granulocyte fraction at a ratio of 1 part hetastarch to 1 part cells, or 1 part hetastarch to 9 parts cells. The resultant mixtures are allowed to settle for 1 hour at room temperature, whereupon the supernatant is withdrawn and the cells suspended therein pelleted by centrifugation. The cell pellets are washed once with PBS and frozen as described in (B) below.

Figure 1 shows a typical flow cytometry profile for fresh (pre-freeze) granulocytes isolated from a normal donor by density gradient centrifugation in Ficoll, followed by hemolysis in ammonium chloride. Briefly, the cells in the upper right-hand quadrant of the figure are granulocytes, as indicated by their large size (high forward scatter) and granularity (high side scatter). The light scatter profile is essentially identical for all three processing methods described above, indicating that none of these processing methods is detrimental to the cells.

B. Cryopreservation of Granulocytes

After processing as described in (A) above, each cell pellet (of granulocytes) is resuspended in 500 uL of chilled (4°C) medium 199 (Gibco) containing 8% (w/v) HSA (Immuno-U.S.), either with or without 100 U/mL DNase (Type I, Sigma) and 100 U/mL heparin (Sigma, H8514). Cryopreservation medium which contains DNase and heparin is hereinafter referred to as "complete cryopreservation medium," while medium lacking DNase and heparin is hereinafter referred to as "incomplete medium."

Twenty microliters are withdrawn and a cell count made using a hemacytometer and a light microscope. The cell concentration is then adjusted to 200×10^6 cells/mL with medium 199 containing 8% HSA. Each cell suspension is diluted to a final concentration of 100×10^6 cells/mL by the dropwise addition, with mixing, of an equal volume of chilled medium 199 containing 15% DMSO (Terra Pharmaceuticals), again either with (complete) or without (incomplete) 100 U/mL DNase and 100 U/mL heparin.

Once mixed, each cell suspension (approximately 1 mL volume) is transferred to a cryovial and placed in the pre-cooled chamber of a controlled-rate freezer. The chamber and the cell suspension are allowed to equilibrate to within 20°C of each other, whereafter the suspension is frozen at a controlled rate as follows: -1°/min until the temperature of the sample is -4°C; -25°/min until the temperature of the chamber is -40°C; -15°/min until the temperature of the chamber is -12°C°, -10/min until the temperature of the chamber is -40°C; -10°/min until the temperature of the chamber is -90°C.

Upon completion of the freezing program, the cryovials are transferred to the vapor phase of liquid nitrogen (LN_2) for storage.

C. Thawing of Granulocytes

Cryovials are removed from liquid N_2 storage and thawed by immersion in a 37°C water bath. As the last ice crystal dissolved, the contents of the cryovial are transferred to a 50 mL centrifuge tube. One milliliter of warmed (37°C) dilution medium is added dropwise, with mixing, to each thawed cell suspension. Dilution medium consists of medium 199, with and without 100 U/mL heparin and 100 U/mL DNase. Cells which are frozen in complete cryopreservation medium are diluted with complete (DNase and heparin-containing) dilution medium, while cells which are frozen with incomplete cryopreservation medium are diluted with incomplete dilution medium. Additional dilution medium is added to each cell suspension, 0.5 mL at a time, with mixing, until the cell suspension is diluted up to a final volume of 10 mL.

The cell suspensions are allowed to sit at room temperature for three hours, at which time the percentage cell loss is determined by counting the number of viable cells remaining and subtracting that number from the number of viable cells frozen to obtain the percentage cell loss post-thaw. As shown in Figure 2(a), post-thaw cell loss is less than about 10% regardless of whether the granulocytes are processed by hemolysis, dextran sedimentation, or starch sedimentation (at two different ratios of cells to starch), provided DNase and heparin are included in the cryopreservation and dilution media. When DNase and heparin are eliminated from the cryopreservation and dilution media, however, cell loss increases 2-3.5 fold (Figure 2(b)).

Figure 3 shows a typical flow cytometry profile for post-thaw granulocytes isolated from a normal donor by density gradient centrifugation and hemolysis, and frozen in complete cryopreservation medium. The light scatter profile (3a) is nearly indistinguishable from that of pre-freeze cells (see Figure 1 for comparison). Propidium iodide staining (3b) indicates that the majority of the thawed cells are viable (compare R1 (viable) to R2 (non-viable cells)).

Hence, it can be concluded from this example that the recovery of viable granulocytes after freezing is improved by the addition of DNase and heparin to the cryopreservation and dilution media. Furthermore, granulocytes can be partially purified prior to freezing by a variety of methods without an appreciable effect on their post-thaw recovery. In this experiment, using a variety of methods to partially purify granulocytes and complete cryopreservation medium, granulocyte recovery was typically at least about 90%.

EXAMPLE 2

BIOLOGICAL ACTIVITY OF POST-THAW GRANULOCYTES ISOLATED FROM NORMAL DONORS AND CRYOPRESERVED IN COMPLETE CRYOPRESERVATION MEDIUM

A. Collection and Processing of Granulocytes

Granulocytes are collected from normal donors as described in Example 1(A) above. In some instances, donors undergo mobilization with G-CSF prior to phlebotomy to increase the number of circulating granulocytes and granulocyte precursors. Table 1 summarizes mobilization and processing data for each of the donors.

Table 1

MOBILIZATION AND PROCESSING OF DONOR GRANULOCYTES

DONOR	MOBILIZATION	PROCESSING
70693	none	hemolysis (2X)#
61493A	G-CSF	hemolysis
61493B	G-CSF	hemolysis
71393K	G-CSF	hemolysis
71393K	G-CSF	hemolysis (2X)#
71493K	G-CSF	dextran

Cells were subjected to two cycles of hemolysis.

B. Cryopreservation of Granulocytes

Granulocytes are cryopreserved in complete cryopreservation medium as described in Example 1(B) above.

C. Biological Activity of Post-Thaw Granulocytes

Granulocytes are thawed as described in Example 1(C) above. After dilution to 10 mL in complete dilution medium, each cell suspension is centrifuged at 800 rpm for 8 minutes in a tabletop centrifuge to pellet the cells. the supernatant is aspirated and discarded, and the pellet is resuspended in an approximately equal volume of PBS containing 100 U/mL heparin, 100 U/mL DNase, and 2% fetal bovine serum (FBS).

The ability of the cells to kill bacteria may be evaluated essentially as described in Baldwin et al., *op. cit.* Briefly, 1 x 10^6 granulocytes in a volume of 100 μL are combined with 600 μL of HBSS and 100 μL 10% human AB serum and allowed to warm to 37°C for 5 minutes. Then 100 μL of *Staphylococcus aureus* bacteria (1 x 10^6) are added, followed by 100 μL of GM-CSF (100 pM final concentration). Aliquots (100 μL) of the mixture are removed at 0-, 30-, 60-, and 120-minute intervals. Each aliquot is serially diluted (10-, 100- and 1000-fold in HBSS) and plated in duplicate on trypticase soy agar. Plates are incubated overnight at 37°C and the number of bacterial colonies formed counted for each time interval and compared to time zero. If the post-thaw granulocytes retain biological activity, they are expected to ingest and destroy a substantial fraction of the bacteria added to the assay. Accordingly, the greater the granulocyte activity, the fewer bacterial colonies one would expect to grow at each time point.

Fresh granulocytes, isolated from non-mobilized donors by density gradient centrifugation on Ficoll followed by dextran sedimentation (as described in Example 1(A)), serve as controls for each assay. The data shown in Table 2 are expressed as percent killing relative to fresh granulocyte controls, where the control is normalized to 100%. In these experiments, the controls actually kill between 57% and 83% of the bacteria in the assay after 2 hours, while the donor (post-thaw) granulocytes actually kill between 23% and 46% of the bacteria in the same time period. When normalized the post-thaw donor granulocytes kill from about 31% to about 79% of the bacteria. For simplicity, only the 2-hour time point is shown in Table 2.

Table 2

BACTERIAL KILLING BY POST-THAW DONOR GRANULOCYTES

DONOR	NORMALIZED PERCENT KILLING
70693	31.5
61493A	31.3
61493B	47.9
71393K	62.6
71393K	56.8
71393K	78.9

The data in Table 2 illustrate that granulocytes mobilized, collected and cryopreserved according to the methods of the instant invention retain at least about 30% of their biological activity post-thaw, relative to unfrozen granulocyte controls, in an intracellular killing assay. When the data in Table 2 are averaged, it can be seen that mobilized donor granulocytes collected and cryopreserved according to the methods of the instant invention retain an average of approximately 51.5% of their biological activity post-thaw relative to fresh, unfrozen control granulocytes.

Post-thaw retention of biological activity does not appear to be affected by prior mobilization with G-CSF, nor does it appear to be affected by the method of processing prior to cryopreservation.

EXAMPLE 3

COLLECTION BY LEUKAPHERESIS AND CRYOPRESERVATION OF GRANULOCYTES FROM G-CSF MOBILIZED DONORS

A. Mobilization and Leukapheresis

G-CSF (5 μg/kg) is administered to a donor about 24 hours prior to apheresis. Approximately seven liters of blood are processed by apheresis on a Cobe Spectra in the presence of low molecular weight HES (1:13 ratio of starch to blood). About 60 billion cells, of which approximately 70% are myeloid cells, collected in a volume of about 250 mL.

B. Cryopreservation

The resultant granulocyte fraction is cryopreserved in complete cryopreservation medium, essentially as described in Example 1(B) above.

C. Thawing of Granulocytes

Granulocytes are thawed in complete dilution medium, essentially as described in Example 1(C) above.

D. Biological Activity of Post-Thaw Granulocytes

The biological activity of post-thaw granulocytes, harvested by apheresis in the presence of 10% pentastarch and cryopreserved according to the methods of the instant invention, may be assessed by an intracellular bacterial killing assay, as described in Example 2(D) above and in Baldwin et al., *op. cit.*, relative to control (fresh unfrozen) granulocytes from a normal, non-mobilized

donor. Control, unfrozen granulocytes kill approximately 86% of the bacteria within 2 hours, while the post-thaw granulocytes kill 64% in the same time period. Normalizing the data to 100%, post-thaw granulocytes retain at least about 75% of their pre-freeze biological activity in an intracellular killing assay, relative to fresh, unfrozen controls.

Hence, the methods of the instant invention can be utilized to collect and cryopreserve mobilized donor granulocytes from an apheresis product, which granulocytes retain at least about 75% of their biological activity, post-thaw in an intracellular killing assay.

EXAMPLE 4

PROPHYLAXIS OF INFECTION IN A GRANULOCYTOPENIC PATIENT BY ADMINISTRATION OF DONOR GRANULOCYTES

To prevent the development of fever and infection in an at-risk patient, granulocytes are harvested from a normal donor of the same HLA type as the patient. In addition, the donor is chosen to be seronegative for CMV, HIV 1, HIV 2, and HBsAg. The donor is also chosen so that the donor's red cells do not agglutinate in the presence of patient serum.

Donors are mobilized with a single subcutaneous administration of G-CSF (300 μg) 12-16 hours before apheresis. Five to seven liters of ACD-A anti-coagulated blood are subjected to apheresis, in the presence of 10% pentastarch to facilitate red cell sedimentation, using a CS3000 Plus Blood Cell Separator (standard protocol available from manufacturer or see Caspar et al., *Blood 81*:2866, 1993). The granulocytapheresis product is recovered in a volume of approximately 250 mL and contains approximately 50 x 10^9 cells.

The pheresis product is allowed to settle by gravity for about one hour at room temperature, after which the granulocyte layer is carefully removed, leaving as many of the red cells behind as possible. The resultant granulocytes are pelleted by centrifugation, the supernatant aspirated and discarded, and the cells resuspended in several volumes of PBS. The cells are again pelleted by centrifugation, the supernatant aspirated and discarded, and the cells resuspended to a concentration of 500 x 10^6 cells/mL in PBS containing 8% HSA, 100 U/mL heparin, and 100 U/mL DNase. The resultant cell suspension is irradiated using a linear accelerator as an e-beam source. A volume of PBS containing 15% DMSO, 100 U/mL heparin, and 100 U/mL DNase, equal to the volume of the cell suspension, is then added to the cell suspension to bring the

final concentration of cells to about 250 x 10^6 cells/mL. The cell suspension is divided into approximately 40 mL aliquots and dump-frozen to about -90°C.

A patient who is at risk of fever or infection due to granulocytopenia (ANC<100 cells/uL) is given a transfusion of donor granulocytes (40 mL, approximately 10 billion cells). A 40 mL aliquot collected and cryopreserved as described above is thawed rapidly by immersion in a 37°C water bath and diluted stepwise with complete dilution medium as described in the examples above. Using a standard blood administration set, one aliquot of granulocytes is transfused into the patient approximately every 48 hours. Transfusions are continued until, in the judgment of the patient's health care provider, the patient is no longer at risk of fever or infection. Generally, this coincides with a self-sustaining ANC >100 cells/uL.

EXAMPLE 5

PROPHYLAXIS OF INFECTION IN A GRANULOCYTOPENIC PATIENT BY ADMINISTRATION OF AUTOLOGOUS GRANULOCYTES

A prospective autologous bone marrow transplant patient is mobilized by the administration of 300 μg of G-CSF 24 hours prior to granulocytapheresis. Approximately 7 liters of ACD-A anti-coagulated blood are processed using a Cobe Spectra and 10% HES. The granulocytes are processed as described in Example 4 except that they are not irradiated prior to freezing.

Granulocyte transfusions are begun on the first day following transplantation of the patient. In addition, the patient receives prophylactic antibiotic therapy and G-CSF. Cryopreserved autologous granulocytes are thawed and diluted as described in Example 4 above. One-fourth aliquot (10 mL pre-dilution volume, about 10^9 cells) is transfused into the patient approximately daily until the patient exhibits a self-sustaining ANC above 100 cells/uL, typically about two to three weeks post-transplant.

EXAMPLE 6

TREATMENT OF A PATIENT WITH FEVER OR INFECTION BY TRANSFUSION OF DONOR GRANULOCYTES

Donor granulocytes are harvested, irradiated, and cryopreserved as described in Example 4 above. A febrile patient is transfused daily with one

aliquot of HLA-matched donor granulocytes (approximately 10 billion cells), thawed and administered as described in Example 4 above. Antibiotics, chosen by the patient's health care provider on the basis of surveillance data and/or culture, and/or gram stain, and/or antibiotic susceptibility data, are co-administered. Transfusions are continued daily until the patient's temperature is persistently less than 38°C.

EXAMPLE 7

COLLECTION BY LEUKAPHERESIS AND CRYOPRESERVATION OF GRANULOCYTES FROM FIVE G-CSF MOBILIZED DONORS

A. Mobilization

Three healthy adult males (subjects 1-3) were mobilized with G-CSF (300 μg, subcutaneous) each day for five days. On the fifth day, each subject received an additional (sixth) dose of G-CSF (300 μg, subcutaneous) approximately 12 hours after their first dose of the day. Each subject underwent leukapheresis 12 hours after the last (sixth) dose of G-CSF.

One healthy, adult female (subject 4) and one healthy, adult male (subject 5) were mobilized with G-CSF (5 μg/kg, subcutaneous) each day for 7 days. The subjects underwent leukapheresis on days 2, 4, 6, and 8.

B. Leukapheresis

In subjects 1, 2, 3, and 5, leukapheresis was performed on an automated, continuous-flow, blood-cells separator, the Cobe Spectra (Lakewood, CO). Between 7 and 12 liters of blood were processed during a 3 to 4 hour time period. Granulocyte collection was facilitated by the use of PENTASPAN pentastarch (DuPont Merck Pharmaceuticals, Wilmington, DE). Twenty-seven mL of Tri-Citrol anticoagulant was added to each 500 mL bottle of PENTASPAN (10% pentastarch in 0.9% NaCl). Each subject received approximately 1300 mL of Tri-Citrol/starch solution during the leukapheresis.

Subject 4 underwent leukapheresis on a Fenwall CS3000 blood separator (Fenwall Laboratories, Deerfield, IL). Granulocyte collection was facilitated by the use of HESPAN hetastarch (DuPont Merck, 6% hetastarch in 0.9% NaCl).

The number of granulocytes collected from each leukapheresis ranged between 96 and 103 billion cells (mean 99 ± 3.6 billion). The hematocrit of the collections ranged from 4% to 23%, with a mean of 13 ± 10%.

C. Cryopreservation

The volume of the granulocytapheresis product ranged from 332 mL to 585 mL, with a mean of 456 ± 127 mL. Granulocyte collections from subjects 1 and 2 were split into 2 equal-sized aliquots. One aliquot from each subject was subjected to irradiation (25 Gy over 1.9 minutes) using a Gamma Cell 1000 (Nordion International, Ontario, Canada), while the other was not.

Granulocyte collections from subjects 1-5 were cryopreserved as follows: the granulocytes were placed in a 600 mL transfer pack (Baxter, Deerfield, IL). Preservative-free sodium heparin (2000 U/mL, Abbott Laboratories, Chicago, IL) was added to the cells to a final concentration of 100 U/mL. Human recombinant DNase (4 mg/mL in 140 mM NaCl/1 mM $CaCl_2$, Genentech, So. San Francisco, CA) was added to a final concentration of 40 ug/mL. The transfer pack was chilled on ice while mixing gently on a rotator table for about 15 minutes. DMSO (Cryoserv, Tera Pharmaceuticals, Salt Lake City, UT) was added slowly (over about 2 minutes) to the pack to bring the final concentration of DMSO to 7.5% (v/v). Fifty mL aliquots (200-250 million cells/mL) were then transferred to 250 mL freeze bags (Cryomed, New Baltimore, MI) and frozen at a rate of -1°C per minute, with compensation for heat release at the phase transition with a burst of liquid nitrogen. The frozen bags were then transferred to liquid nitrogen for storage.

D. Thawing of Granulocytes

At various time intervals after freezing, bags of granulocytes were removed from liquid nitrogen and thawed by immersion in a 37°C water bath until the last ice crystal dissolved. The thawed cells were diluted slowly with RPMI (49 mL per 1 mL of cells, BioWhittaker, Walkersville, MD), containing 2 mM glutamine (BioWhittaker), 10 U/mL heparin, and 10% Type O human plasma (Red Cross, Portland, OR). The plasma was recalcified prior to use by adding heparin to 20 U/mL and $CaCl_2$ (2.5 M in deionized water, J.T. Baker, Phillipsburg, NJ) to a final concentration of 2.5 mM.

Upon thawing, a mean of 97 ± 6% of the cells initially frozen was recovered. Mean viability immediately after thawing was 89 ± 3%. Mean viability 3 hours post-thaw was 70 ± 5%.

E. Biological Activity of Post-Thaw Granulocytes

The biological activity of the cells after thawing was assessed by means of a chemiluminescence assay of repiratory burst activity. Briefly, one million thawed cells (approximately 1 mL) were placed in polystyrene chemiluminescence cuvettes (Analytical Luminescence Laboratory, San Diego, CA). The cuvettes were filled with RPMI containing 10mM Hepes (BioWhittaker, Walkersville, MD) and 15ug/mL HSA. The assay was initiated by adding luminol to a final concentration of 10 uM and PMA (Sigma, St. Louis, MO) to a final concentration of 1 uM to the reaction mixture. Ten-second readings were taken at 1, 5, 15, 30, 60, and 120 minutes after initiation of the reaction, using a Monolight 2001 luminometer (Analytical Luminescence Laboratory, San Diego, CA) set on integration mode. No clumping was detected in any of the samples during the course of assay.

Median respiratory burst activity for irradiated granulocytes post-thaw was 19 ± 6% (range 10-24% n=4) and for non-irradiated granulocytes post-thaw was 18.5 ± 13% (range 9-28%, n=2) , relative to fresh (unfrozen) granulocytes.

Plastic adherence was evaluated as follows. Cells were plated onto Permanox dual chamber slides. Approximately $2x10^6$ cells in 1 mL of RPMI, containing 10% human plasma and 10 U/mL heparin, were added to each chamber. The slides were incubated at 37°C in a humidified atmosphere containing 5% CO_2. At various intervals, aliquots were removed and counted in a polypropylene snap cap tube on a Coulter Counter to determine the number of cells still in suspension. The number of adherent cells was determined by staining with acridine orange/ethidium bromide, followed by fluorescence microscopic examination.

Cells remained greater than 95% viable during the three hour course of the plastic adherence assays. Plastic adherence values increased for post-thaw granulocytes relative to fresh (unfrozen) granulocytes, but were not affected by whether or not the cells had been irradiated prior to freezing. Post-thaw values averaged 162% of fresh cells. Adherence is believed to be a measure of the degree to which granulocytes are activated. Hence, post-thaw granulocytes appear to be activated relative to fresh granulocytes inasmuch as a higher percentage of the post-thaw cells adhere to plastic under the conditions employed.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

Claims

1. A composition, comprising frozen granulocytes in a cryopreservation medium, said granulocytes having a biological activity upon thawing of at least 30% as compared to fresh donor granulocytes in an intracellular killing assay.

2. A composition, comprising frozen mobilized granulocytes in a cryopreservation medium, said granulocytes having a biological activity upon thawing of at least 30% as compared to fresh donor granulocytes in an intracellular killing assay.

3. The composition according to claim 1 or 2 wherein said granulocytes have a biological activity upon thawing of at least 50% as compared to fresh donor granulocytes in an intracellular killing assay.

4. A composition comprising frozen granulocytes in a cryopreservation medium, said granulocytes having a biological activity upon thawing of at least 10% as compared to fresh granulocytes in a respiratory burst activity assay.

5. The composition according to any one of claims 1, 2 or 4 wherein said granulocytes are frozen at a concentration of between 250 x 10^6 and 500 x 10^6 cells/mL.

6. The composition according to any one of claims 1, 2 or 4 wherein said granulocytes have been irradiated.

7. The composition according to any one of claims 1, 2 or 4 wherein said cryopreservation medium comprises a penetrating cryoprotectant, an anticoagulant, and a nuclease.

8. The composition according to claim 7 further comprising a physiologically acceptable medium selected from the group consisting of phosphate buffered saline, RPMI, DMEM, and medium 199.

9. The composition according to claim 7 wherein said penetrating cryoprotectant is DMSO.

10. The composition according to claim 9 wherein said DMSO is present at a concentration of 5% to 10%.

11. The composition according to claim 9 wherein said DMSO is present at a concentration of 7.5%.

12. The composition according to claim 7 further comprising a source of protein.

13. The composition according to claim 7 wherein said anticoagulant is selected from the group consisting of acid citrate dextrose, EDTA and heparin.

14. The composition according to claim 13 wherein said heparin is present at a concentration of between 50 and 150 units/mL.

15. The composition according to claim 14 wherein said heparin is present at a concentration of 100 units/mL.

16. The composition according to claim 7 wherein said nuclease is a DNase.

17. The composition according to claim 16 wherein said DNase is a type I or type II DNase.

18. The composition according to claim 16 wherein said DNase is a recombinant human DNase.

19. The composition according to claim 16 wherein said DNase is present at a concentration of between 50 and 150 units/mL.

20. The composition according to claim 16 wherein said DNase is present at a concentration of 100 units/mL.

21. A method of preserving granulocytes, comprising:

(a) harvesting granulocytes from the blood of a patient or donor by apheresis; and

(b) freezing the harvested granulocytes in a cryopreservation medium, such that, upon thawing, said granulocytes have a biological activity of at least 30% as compared to fresh granulocytes in an intracellular killing assay.

22. A method of preserving granulocytes, comprising:

(a) harvesting granulocytes from the blood of a patient or donor by apheresis; and

(b) freezing the harvested granulocytes in a cryopreservation medium, such that, upon thawing, said granulocytes have a biological activity of at least 10% as compared to fresh granulocytes in a respiratory burst assay.

23. A method of preserving granulocytes, comprising:

(a) harvesting granulocytes from the blood of a patient or donor by apheresis; and

(b) freezing the harvested granulocytes in a cryopreservation medium, such that, upon thawing, said granulocytes have a biological activity of at least 30% as compared to fresh granulocytes in an intracellular killing assay, or a biological activity of at least 10% as compared to fresh granulocytes in a respiratory burst assay.

24. The method according to any one of claims 21 to 23, further comprising, prior to the step of harvesting, mobilizing granulocytes in the patient or donor .

25. The method according to claim 24 wherein said granulocytes are mobilized by administration of a cytokine to said patient or donor.

26. The method according to claim 25 wherein said cytokine is selected from the group consisting of G-CSF, GM-CSF, IL-3, SCF and IL-1.

27. The method according to claim 24 wherein said granulocytes are mobilized by administration of a steroid to said patient or donor.

28. The method according to claim 27 wherein said steroid is selected from the group consisting of prednisone, dexamethasone, etiocholanolone and hydrocortisone.

29. The method according to claim 24 wherein said granulocytes are mobilized by administration of a cytokine and a steroid to said patient or donor.

30. The method according to any one of claims 21 to 23, further comprising, subsequent to the step of harvesting granulocytes, partially purifying the harvested granulocytes.

31. The method according to any one of claims 21 to 23 wherein said granulocytes are harvested in the presence of a red cell sedimenting agent selected from the group consisting of hydroxyethyl starch, pentastarch, fluid gelatin and dextran.

32. The method according to any one of claims 21 to 23 wherein said granulocytes are frozen at a concentration of between 250 x 10^6 and 500 x 10^6 cells/mL.

33. The method according to any one of claims 21 to 23 wherein said granulocytes are irradiated prior to the step of freezing.

34. The method according to any one of claims 21 to 23 wherein said cryopreservation medium comprises a penetrating cryoprotectant, an anticoagulant, and a nuclease.

35. The method according to claim 34 wherein said cryopreservation medium further comprises a physiologically acceptable medium selected from the group consisting of phosphate buffered saline, RPMI, DMEM, and medium 199.

36. The method according to claim 34 wherein said penetrating cryoprotectant is DMSO.

37. The method according to claim 36 wherein said DMSO is present at a concentration of 5% to 10%.

38. The method according to claim 36 wherein said DMSO is present at a concentration of 7.5%.

39. The method according to claim 34 wherein said cryopreservation medium further comprises a source of protein.

40. The method according to claim 34 wherein said anticoagulant is selected from the group consisting of acid citrate dextrose, EDTA and heparin.

41. The method according to claim 40 wherein said heparin is present at a concentration of between 50 and 150 units/mL.

42. The method according to claim 40 wherein said heparin is present at a concentration of 100 units/mL.

43. The method according to claim 34 wherein said nuclease is a DNase.

44. The method according to claim 43 wherein said DNase is a type I or type II DNase.

45. The method according to claim 43 wherein said DNase is a recombinant human DNase.

46. The method according to claim 43 wherein said DNase is present at a concentration of between 50 and 150 units/mL.

47. The method according to claim 43 wherein said DNase is present at a concentration of 100 units/mL.

48. A method for preventing fever or infection in a patient during a period of risk for fever or infection, comprising:

(a) harvesting granulocytes from the blood of a patient or donor by apheresis;

(b) freezing the harvested granulocytes in a cryopreservation medium, such that, upon thawing, the granulocytes have a biological activity of at least 30% as compared to fresh granulocytes in an intracellular killing assay;

(c) thawing said frozen granulocytes; and

(d) administering a prophylactically effective amount of thawed granulocytes to a patient during a period of risk for fever or infection, such that said fever or infection may be prevented.

49. A method for preventing fever or infection in a patient during a period of risk for fever or infection, comprising:

(a) harvesting granulocytes from the blood of a patient or donor by apheresis;

(b) freezing the harvested granulocytes in a cryopreservation medium, such that, upon thawing, the granulocytes have a biological activity of at least 10% as compared to fresh granulocytes in a respiratory burst assay;

(c) thawing said frozen granulocytes; and

(d) administering a prophylactically effective amount of thawed granulocytes to a patient during a period of risk for fever or infection, such that said fever or infection may be prevented.

50. A method for preventing fever or infection in a patient during a period of risk for fever or infection, comprising:

(a) harvesting granulocytes from the blood of a patient or donor by apheresis;

(b) freezing the harvested granulocytes in a cryopreservation medium, such that, upon thawing, the granulocytes have a biological activity of at least 30% as compared to fresh granulocytes in an intracellular killing assay, or a biological activity of at least 10% as compared to fresh granulocytes in a respiratory burst assay;

(c) thawing said frozen granulocytes; and

(d) administering a prophylactically effective amount of thawed granulocytes to a patient during a period of risk for fever or infection, such that said fever or infection may be prevented.

51. A method for treating fever or infection in a patient during a period of risk for fever or infection, comprising:

(a) harvesting granulocytes from the blood of a patient or donor by apheresis;

(b) freezing the harvested granulocytes in a cryopreservation medium, such that, upon thawing, the granulocytes have a biological activity of at least 30% as compared to fresh granulocytes in an intracellular killing assay;

(c) thawing said frozen granulocytes; and

(d) administering a therapeutically effective amount of thawed granulocytes to a patient during a period of fever or infection, such that said fever or infection may be reduced.

52. A method for treating fever or infection in a patient during a period of risk for fever or infection, comprising:

(a) harvesting granulocytes from the blood of a patient or donor by apheresis;

(b) freezing the harvested granulocytes in a cryopreservation medium, such that, upon thawing, the granulocytes have a biological activity of at least 10% as compared to fresh granulocytes in a respiratory burst assay;

(c) thawing said frozen granulocytes; and

(d) administering a therapeutically effective amount of thawed granulocytes to a patient during a period of fever or infection, such that said fever or infection may be reduced.

53. A method for treating fever or infection in a patient during a period of risk for fever or infection, comprising:

(a) harvesting granulocytes from the blood of a patient or donor by apheresis;

(b) freezing the harvested granulocytes in a cryopreservation medium, such that, upon thawing, the granulocytes have a biological activity of at least 30% as compared to fresh granulocytes in an intracellular killing assay, or a biological activity of at least 10% as compared to fresh granulocytes in a respiratory burst assay;

(c) thawing said frozen granulocytes; and

(d) administering a therapeutically effective amount of thawed granulocytes to a patient during a period of fever or infection, such that said fever or infection may be reduced.

54. The method according to any one of claims 48 to 53, further comprising, subsequent to the step of harvesting granulocytes, partially purifying the harvested granulocytes.

55. The method according to any one of claims 48 to 53, further comprising, subsequent to the step of harvesting granulocytes, aliquoting the granulocytes.

56. The method according to any one of claims 48 to 53 wherein said patient is a granulocytopenic patient.

57. The method according to any one of claims 48 to 53 wherein said patient is a patient with granulocyte dysfunction.

58. The method according to any one of claims 48 to 53 wherein said patient is a patient with an idiopathic or hereditary defect in granulocyte number or function.

59. The method according to any one of claims 48 to 53 wherein said patient is a bone marrow transplant patient.

60. The method according to any one of claims 48 to 53 wherein said patient is a patient on a myelosuppressive chemotherapeutic regimen.

61. The method according to any one of claims 48 to 53 wherein said patient is a patient with sepsis.

62. The method according to any one of claims 48 to 53 wherein said patient is a burn patient.

63. The method according to any one of claims 48 to 53 wherein said patient is a peripheral blood stem cell transplant patient.

64. The method according to any one of claims 48 to 53 wherein said patient experienced blood loss.

65. The method according to any one of claims 48 to 53 wherein said patient is a radiation therapy patient.

66. The method according to any one of claims 48 to 50, further comprising, prior to the step of harvesting granulocytes, mobilizing granulocytes in the patient or donor.

67. The method according to any one of claims 51 to 53, further comprising, prior to the step of harvesting granulocytes, mobilizing granulocytes in the patient or donor.

68. The method according to claim 66 wherein said granulocytes are mobilized in a patient or donor by administration of a cytokine to said patient or donor.

69. The method according to claim 67 wherein said granulocytes are mobilized in a patient or donor by administration of a cytokine to said patient or donor.

70. The method according to claim 68 wherein said cytokine is selected from the group consisting of G-CSF, GM-CSF, IL-3, SCF, and IL-1.

71. The method according to claim 69 wherein said cytokine is selected from the group consisting of G-CSF, GM-CSF, IL-3, SCF, and IL-1.

72. The method according to claim 66 wherein said granulocytes are mobilized in a patient or donor by administration of a steroid.

73. The method according to claim 67 wherein said granulocytes are mobilized in a patient or donor by administration of a steroid.

74. The method according to claim 72 wherein said steroid is selected from the group consisting of prednisone, dexamethasone, etiocholanolone and hydrocortisone.

75. The method according to claim 73 wherein said steroid is selected from the group consisting of prednisone, dexamethasone, etiocholanolone and hydrocortisone.

76. The method according to claim 66 wherein said granulocytes are mobilized in a donor by administration of a cytokine and a steroid to said patient or donor.

77. The method according to claim 67 wherein said granulocytes are mobilized in a donor by administration of a cytokine and a steroid to said patient or donor.

78. The method according to any one of claims 48 to 53 wherein said granulocytes are harvested in the presence of a red cell sedimenting agent selected from the group consisting of hydroxyethyl starch, pentastarch, fluid gelatin and dextran.

79. The method according to any one of claims 48 to 53 wherein said granulocytes are frozen at a concentration of between 250 x 10^6 and 500 x 10^6 cells/mL.

80. The method according to any one of claims 48 to 53 wherein said granulocytes are irradiated.

81. The method according to any one of claims 48 to 53 wherein said cryopreservation medium comprises a penetrating cryoprotectant, an anticoagulant, and a nuclease.

82. The method according to claim 81 wherein said cryopreservation medium further comprises a physiologically acceptable medium selected from the group consisting of phosphate buffered saline, RPMI, DMEM, and medium 199.

83. The method according to claim 81 wherein said penetrating cryoprotectant is DMSO.

84. The method according to claim 83 wherein said DMSO is present at a concentration of 5% to 10%.

85. The method according to claim 83 wherein said DMSO is present at a concentration of 7.5%.

86. The method according to claim 81 wherein said cyropreservation medium further comprises a source of protein.

87. The method according to claim 81 wherein said anticoagulant is selected from the group consisting of acid citrate dextrose, EDTA and heparin.

88. The method according to claim 83 wherein said heparin is present at a concentration of between 50 and 150 units/mL.

89. The method according to claim 83 wherein said heparin is present at a concentration of 100 units/mL.

90. The method according to claim 81 wherein said nuclease is a DNase.

91. The method according to claim 90 wherein said DNase is a type I or type II DNase.

92. The method according to claim 90 wherein said DNase is a recombinant human DNase.

93. The method according to claim 90 wherein said DNase is present at a concentration of between 50 and 150 units/mL.

94. The method according to claim 90 wherein said DNase is present at a concentration of 100 units/mL.

95. The method according to any one of claims 48 to 53 wherein said granulocytes are allogeneic to said patient.

96. The method according to any one of claims 48 to 53 wherein said granulocytes are autologous to said patient.

97. The method according to any one of claims 48 to 53 wherein said granulocytes are syngeneic to said patient.

98. The method according to any one of claims 48 to 53, further comprising, administering to said patient a composition selected from the group consisting of antibiotics, antifungals, antivirals, and antiprotozoals.

99. Granulocytes characterized as having been prepared by the process comprising:

(a) harvesting granulocytes from the blood of a patient or donor by apheresis;

(b) freezing the harvested granulocytes in a cryopreservation medium, such that, upon thawing, the granulocytes have a biological activity of at least 30% as compared to fresh granulocytes in an intracellular killing assay; and

(c) thawing said frozen granulocytes.

100. Granulocytes characterized as having been prepared by the process comprising:

(a) harvesting granulocytes from the blood of a patient or donor by apheresis;

(b) freezing the harvested granulocytes in a cryopreservation medium, such that, upon thawing, the granulocytes have a biological activity of at least 10% as compared to fresh granulocytes in a respiratory burst activity assay; and

(c) thawing said frozen granulocytes.

101. Granulocytes characterized as having been prepared by the process comprising:

(a) harvesting granulocytes from the blood of a patient or donor by apheresis;

(b) freezing the harvested granulocytes in a cryopreservation medium, such that, upon thawing, the granulocytes have a biological activity of at least 30% as compared to fresh granulocytes in an intracellular killing assay, or a biological activity of at least 10% as compared to fresh granulocytes in a respiratory burst assay; and

(c) thawing said frozen granulocytes.

102. The granulocytes according to any one of claims 99 to 101, further comprising, prior to the step of harvesting, mobilizing granulocytes in said patient or donor.

103. The granulocytes according to claim 102 wherein said granulocytes are mobilized by administration of a cytokine to said patient or donor.

104. The granulocytes according to claim 103 wherein said cytokine is selected from the group consisting of G-CSF, GM-CSF, IL-3, SCF and IL-1.

105. The granulocytes according to claim 102 wherein said granulocytes are mobilized by administration of a steroid to said patient or donor.

106. The granulocytes according to claim 105 wherein said steroid is selected from the group consisting of prednisone, dexamethasone, etiocholanolone and hydrocortisone.

107. The method according to claim 102 wherein said granulocytes are mobilized by administration of a cytokine and a steroid to said patient or donor.

108. The granulocytes according to claim any one of claims 99-101, further comprising, subsequent to the step of harvesting granulocytes, partially purifying the harvested granulocytes.

109. The granulocytes according to claim any one of claims 99-101 wherein said granulocytes are frozen at a concentration of between 250 x 10^6 and 500 x 10^6 cells/mL.

110. The granulocytes according to claim any one of claims 99-101 wherein said granulocytes are irradiated.

111. The granulocytes according to claim any one of claims 99-101 wherein said cryopreservation medium comprises a penetrating cryoprotectant, an anticoagulant, and a nuclease.

112. The granulocytes according to claim 111 wherein said cryopreservation medium further comprises a physiologically acceptable medium selected from the group consisting of phosphate buffered saline, RPMI, DMEM, and medium 199.

113. The granulocytes according to claim 111 wherein said penetrating cryoprotectant is DMSO.

114. The granulocytes according to claim 113 wherein said DMSO is present at a concentration of 5% to 10%.

115. The granulocytes according to claim 113 wherein said DMSO is present at a concentration of 7.5%.

116. The granulocytes according to claim 113 wherein said cryopreservation medium further comprises a source of protein.

117. The granulocytes according to claim 111 wherein said anticoagulant is selected from the group consisting of acid citrate dextrose, EDTA and heparin.

118. The granulocytes according to claim 131 wherein said heparin is present at a concentration of between 50 and 150 units/mL.

119. The granulocytes according to claim 111 wherein said heparin is present at a concentration of 100 units/mL.

120. The granulocytes according to claim 111 wherein said nuclease is a DNase.

121. The granulocytes according to claim 120 wherein said DNase is a type I or type II DNase.

122. The granulocytes according to claim 120 wherein said nuclease is a recombinant human DNase.

123. The granulocytes according to claim 120 wherein said DNase is present at a concentration of between 50 and 150 units/mL.

124. The granulocytes according to claim 120 wherein said DNase is present at a concentration of 100 units/mL.

125. The granulocytes according to claim any one of claims 99 to 101 wherein said granulocytes are harvested in the presence of a red cell sedimenting agent selected from the group consisting of hydroxyethyl starch, pentastarch, fluid gelatin and dextran.

126. A composition, comprising a penetrating cryoprotectant, an anticoagulant, and a nuclease.

127. The composition according to claim 126 further comprising a physiologically acceptable medium selected from the group consisting of phosphate buffered saline, RPMI, DMEM, and medium 199.

128. The composition according to claim 126 wherein said penetrating cryoprotectant is DMSO.

129. The composition according to claim 128 wherein said DMSO is present at a concentration of 5% to 10%.

130. The composition according to claim 128 wherein said DMSO is present at a concentration of 7.5%.

131. The composition according to claim 126 further comprising a source of protein.

132. The composition according to claim 126 wherein said anticoagulant is selected from the group consisting of acid citrate dextrose, EDTA and heparin.

133. The composition according to claim 132 wherein said heparin is present at a concentration of between 50 and 150 units/mL.

134. The composition according to claim 133 wherein said heparin is present at a concentration of 100 units/mL.

135. The composition according to claim 126 wherein said nuclease is a DNase.

136. The composition according to claim 135 wherein said DNase is a type I or type II DNase.

137. The composition according to claim 135 wherein said DNase is a recombinant human DNase.

138. The composition according to claim 135 wherein said DNase is present at a concentration of between 50 and 150 units/mL.

139. The composition according to claim 135 wherein said DNase is present at a concentration of 100 units/mL.

140. A composition comprising:

(a) white cells which are at least 50% granulocytes by number, said granulocytes having a biological activity of at least 30% as compared to fresh granulocytes;

(b) a penetrating cryoprotectant; and

(c) a nuclease.

141. A composition comprising:

(a) white cells which are at least 50% granulocytes by number, said granulocytes having a biological activity of at least 10% as compared to fresh granulocytes in a respiratory burst activity assay;

(b) a penetrating cryoprotectant; and

(c) a nuclease.

142. A composition comprising:

(a) white cells which are at least 50% granulocytes by number, said granulocytes having a biological activity of at least 30% as compared to fresh granulocytes, or a biological activity of at least 10% as compared to fresh granulocytes in a respiratory burst activity assay;

(b) a penetrating cryoprotectant; and

(c) a nuclease.

143. The composition according to any one of claims 140 to 142, further comprising an anticoagulant.

144. The composition according to claim 141 wherein said anticoagulant is heparin.

145. The composition according to any one of claims 140 to 142 wherein said penetrating cryoprotectant is DMSO.

146. The composition according to claim 145 wherein said DMSO is present at a concentration of between 5% and 10%.

148. The composition according to any one of claims 140 to 142, further comprising a red cell sedimenting agent.

149. The composition according to any one of claims 140 to 142 wherein said nuclease is a DNase.

150. The composition according to any one of claims 140 to 142 wherein said granulocytes are mobilized granulocytes.

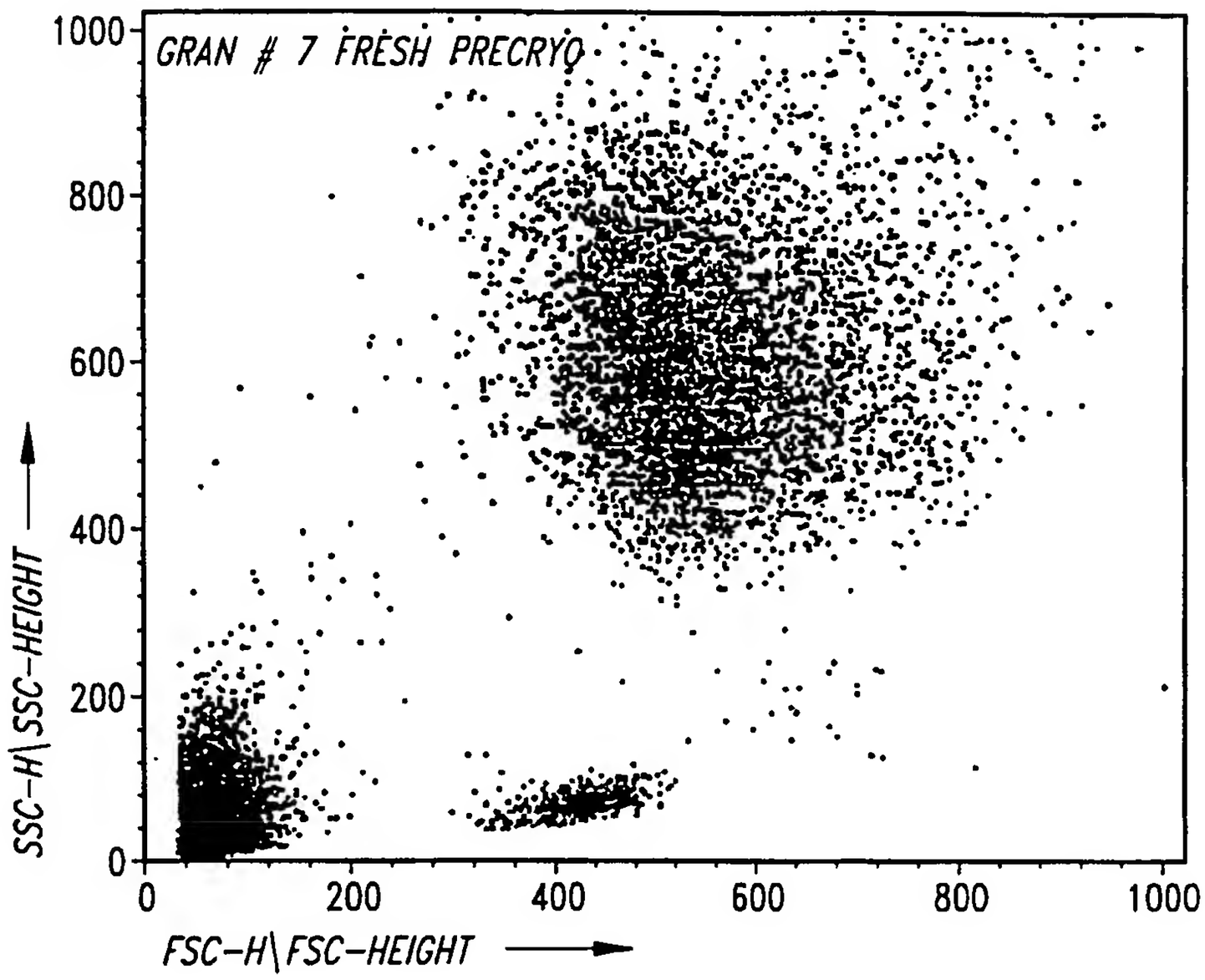
GRAN # 7 FRESH PRECRYO
1000
800
600
400
200
0
0
200
400
600
800
1000
SSC-H\SSC-HEIGHT
FSC-H\FSC-HEIGHT

Fig. 1

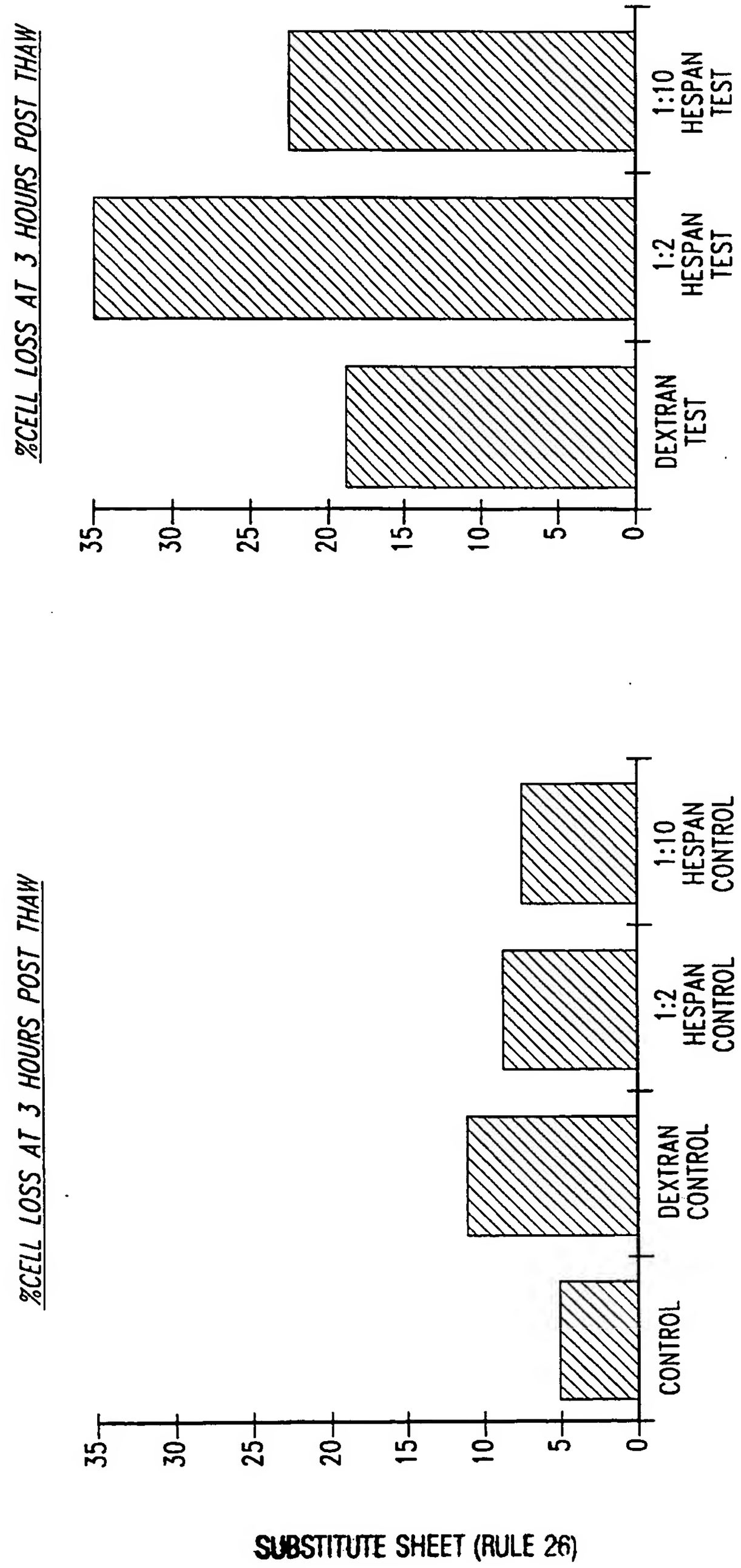
%CELL LOSS AT 3 HOURS POST THAW
35
30
25
20
15
10
5
0
1:10 HESPAN TEST
1:2 HESPAN TEST
DEXTRAN TEST

Fig. 2b

%CELL LOSS AT 3 HOURS POST THAW
35
30
25
20
15
10
5
0
1:10 HESPAN CONTROL
1:2 HESPAN CONTROL
DEXTRAN CONTROL
CONTROL

Fig. 2a

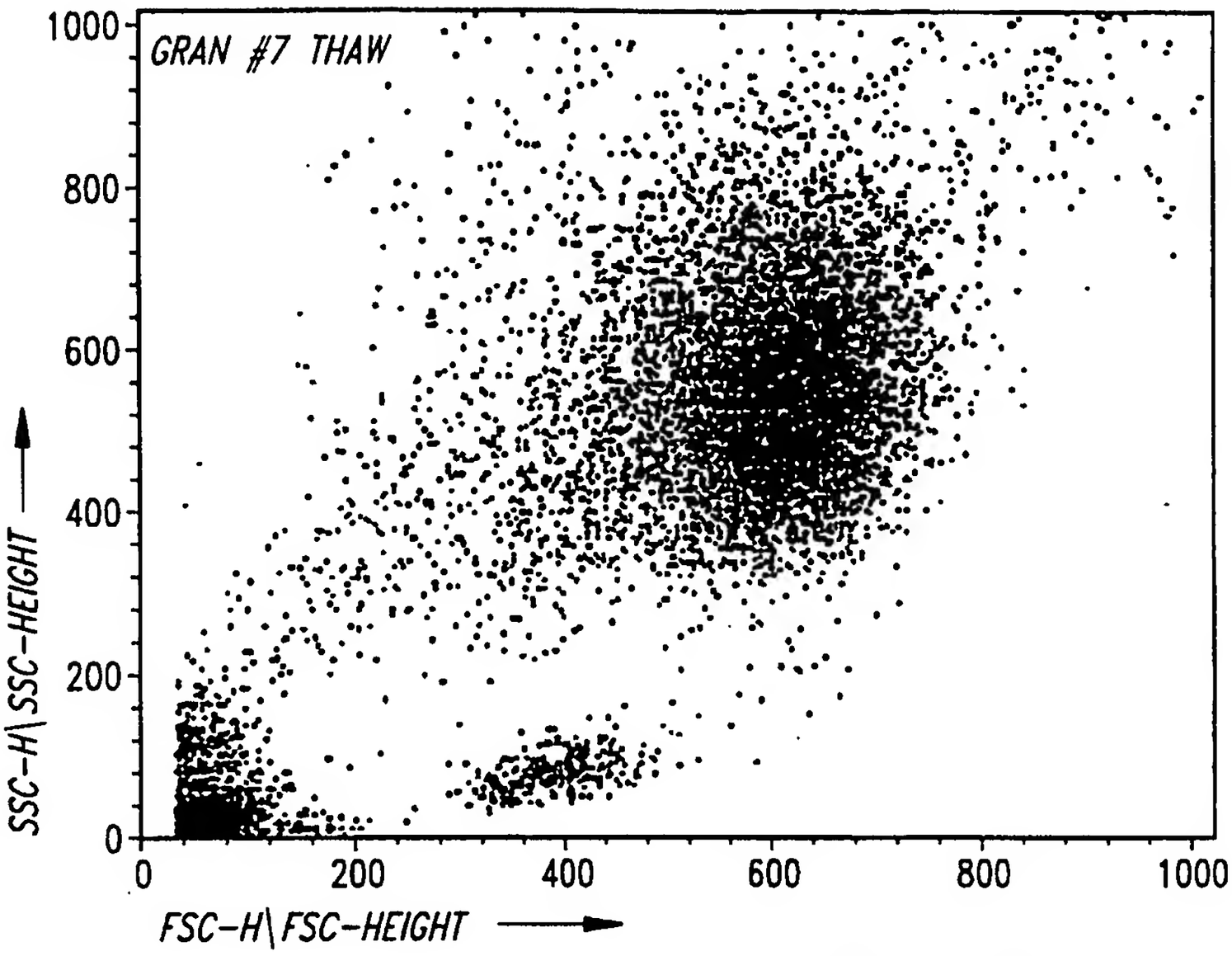
GRAN #7 THAW
1000
800
600
400
200
0
0
200
400
600
800
1000
SSC-H\SSC-HEIGHT
FSC-H\FSC-HEIGHT

Fig. 3A

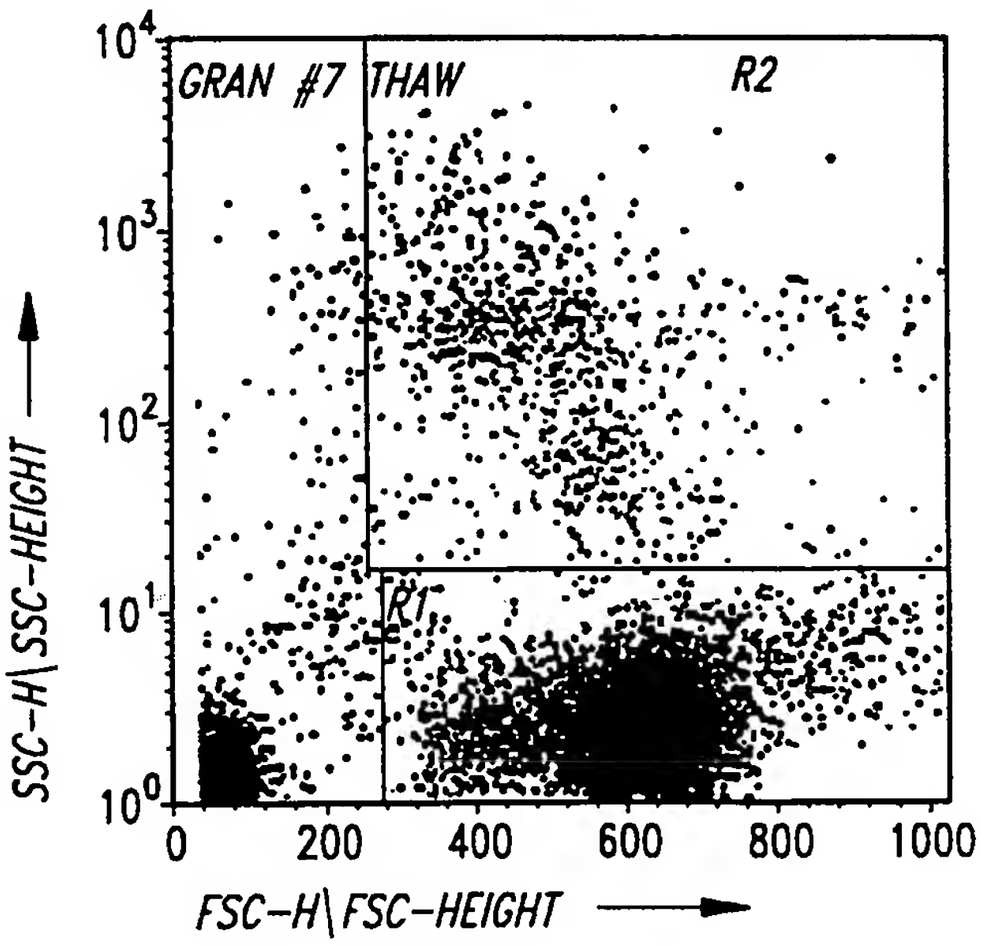
GRAN #7
THAW
R2
R1
10^4
10^3
10^2
10^1
10^0
0
200
400
600
800
1000
SSC-H\SSC-HEIGHT
FSC-H\FSC-HEIGHT

Fig. 3B

INTERNATIONAL SEARCH REPORT

Intern. ..al Application No
PCT/US 94/11735

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K35/14 A01N1/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CRYOBIOLOGY, vol.18, no.6, December 1981, ROCKVILLE, MD., US pages 533 - 540 R.S. HILL ET AL. 'IMPROVED FUNCTIONAL RECOVERY OF HUMAN GRANULOCYTES AFTER CRYOPRESERVATION.' cited in the application see page 534, left column, line 5 - line 17; table 1 see page 534, left column, line 29 - line 32 see page 540, left column, line 5 - line 23 --- -/--	1-150

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 January 1995	06.02.95
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Ryckebosch, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern. nal Application No
PCT/US 94/11735

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,93 18136 (CYTOMED, INC.) 16 September 1993 see page 2, line 15 - line 18; claims 1,2,6,7 see page 7, line 8 - line 13 see page 8, line 17 - line 25	1-5, 7-32, 34-79, 81-109, 111-150
Y	WO,A,93 07745 (CELLPRO, INCORPORATED) 29 April 1993 see page 7, line 23 - page 9, line 24; claims 1-14	1-5, 7-32, 34-79, 81-109, 111-150
Y	R.NETA 'RADIATION, EFFECTS ON IMMUNE SYSTEM, IN I.M. ROITT (ED.) "ENCYCLOPEDIA OF IMMUNOLOGY"' 1992 , ACADEMIC PRESS , LONDON, GB see pages1298-1301, particularly page 1299, left-hand column, lines 14-16	6,33,80, 110
A	US,A,4 004 975 (F.J. LIONETTI ET AL.) 25 January 1977 see the whole document	1-150
A	DE,A,29 29 278 (FORSCHUNGSGESELLSCHAFT FÜR BIOMEDIZINISCHE TECHNIK E.V.) 29 January 1981 see page 9, line 13 - line 19; claims 1-4,16,17; examples 2,9	1-150

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/11735

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 48-98 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern. nal Application No
PCT/US 94/11735

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9318136	16-09-93	NONE	
WO-A-9307745	29-04-93	CA-A- 2122140	29-04-93
		EP-A- 0609379	10-08-94
US-A-4004975	25-01-77	NONE	
DE-A-2929278	29-01-81	NONE	